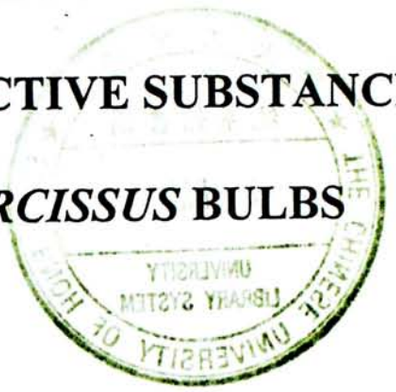
BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF NARCICLASINE, A BIOACTIVE SUBSTANCE ISOLATED FROM *NARCISSUS* BULBS



by

Bi Yu Rong

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Graduate school

The Chinese University of Hong Kong

Thesis Committee

Dr. K.H.Yung (Supervisor)

Dr. H.S.Kwan (Internal Examiner)

Dr. Y.S.Wong (Internal Examiner)

Prof. S.F.Yang (External Examiner)

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ABSTRACT

Narciclasine (NCS) is one of the many bioactive substances that have been isolated from the bulbs of Amaryllidaceae and characterised. In this study, the presence of NCS in the mucilage secreted from *Narcissus* bulbs was confirmed. Effects of NCS on seed germination and seedling growth were described and compared with those induced by abscisic acid (ABA). Both NCS and ABA had very similar action. They inhibited seed germination and seedling growth in a dose-depended manner. In both kinds of inhibition, the activity of NCS was higher than those of ABA at the higher concentration (10^{-5}M). The simultaneous incubation of NCS with ABA (10^{-6}M) did not increase the inhibition of NCS on seed germination and seedling growth.

Effects of NCS on the action of phytohormones, indole-3-acetic acid (IAA), gibberellic acid (GA_3) and benzyladenine (BA) were also studied. At 10^{-6}M and 10^{-5}M concentrations, NCS significantly inhibited IAA activity in the wheat coleoptile section test. IAA (10^{-5}M) could partially reverse the inhibitory effect of 10^{-7}M NCS. BA activities were also strongly inhibited by NCS at a concentration of 10^{-6}M and above in both the expanding and greening of excised radish cotyledons. GA_3-induced α-amylase activity in the barley endosperm was markedly blocked by NCS (10^{-6} - 10^{-5}M). These results suggest that NCS may be a general seed germination inhibitor and an anti-IAA substance. NCS may also block the GA activity and BA activity.

A treatment with NCS affected growth and plastid development of excised radish cotyledons. The inhibitory effect of NCS on growth and greening of excised radish cotyledons could not be prevented by simultaneous addition of BA. The inhibitory effects of NCS on the growth and greening of excised radish cotyledons could be reversed by 10^{-5}M BA if the incubation in NCS was not more than 4 hr. The incubation in water for 12 or 24 hr, followed by being transferred to NCS solutions, could reduce the inhibitory effect of NCS on the growth of excised radish cotyledons, especially incubation in water for 24 hr. In the chlorophyll formation, only the preincubation in water for 24 hr could reduce the inhibition of NCS after they were transferred to NCS solutions for 12 hr. However, when the cotyledons were incubated in NCS solutions for 24 hr, the chlorophyll contents of excised radish cotyledons decreased. When the excised cotyledons were preincubated in water in dark, the inhibitory effect of NCS on the growth of those cotyledons was also markedly reduced. But their greening was still strongly inhibited by 10^{-6}M and 10^{-5} M NCS as in those cotyledons without preincubation with water in the dark. These findings suggest that NCS probably not only inhibited chlorophyll production of excised radish cotyledons grown in light, but also induced the degradation of formed chlorophyll.

From ultrastructural studies, NCS significantly inhibited the degradation of protein bodies and lipid bodies, as well as chloroplast formation of the excised radish cotyledons after 48 hr in the light. There was only a little degradation of protein bodies and lipid bodies and almost no chloroplast formation in the excised radish cotyledons treated with NCS (10^{-6}M and 10^{-5}M). The degradation of protein bodies and lipid bodies decreased with increasing NCS concentrations. In the time course study, a significant number of chloroplasts appeared; however, no typical granum structure was established at 24 hr. A

large vacuole and chloroplasts appeared in the center of the cell and near the cell wall up to 48 hr, respectively. The extent of chloroplast formed was well developed. The cotyledons preincubated in water for 48 hr in dark showed obvious degradation of protein and lipid bodies, and some etioplasts with large starch appeared. When the excised cotyledons were exposed to light for 12 hr, grana formed in the plastids of control cotyledons. There were almost no plastid developing and no grana forming in the excised cotyledons after they were transferred to NCS (10^{-6}M and 10^{-5}M) in light up to 24 hr. These results suggest that either the breakdown of reserves or the development of photosynthetic organs was significantly inhibited by NCS, whereas NCS was more effective in inhibiting the chloroplast differentiation and chlorophyll synthesis.

NCS (10^{-7}M) demonstrated marked inhibitory effects on the accumulation of δ-aminolevulinic acid (ALA) and chlorophyll. NCS (10^{-6}M) completely blocked ALA accumulation after 24 hr in the light. It also suggested that NCS might be a protein synthesis inhibitor in chlorophyll synthesis in light similar to the inhibition on GA-induced α-amylase production in barley half-seed. The inhibitory effect of NCS on chlorophyll synthesis of etiolated wheat leaves exposed to light possibly blocked ALA synthase activity.

NCS could block isocitrate lyase and hydroxypyruvate reductase activity during a period of growth in light for 48 hr. NCS (10^{-5}M) completely inhibited the activities of both enzymes. These inhibitions were either on the activity of the enzyme directly and/or the synthesis of this enzyme.

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List of abbreviations

ABA	Abscisic acid
AcOEt	Ethyl acetate
ALA	δ-Aminolevulinic acid
BA	Benzyladenine
Chl	Chlorophyll
Chl a	Chlorophyll a
Chl b	Chlorophyll b
DMAB	p-Dimethylaminobenzaldehyde
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
GA	Gibberellin
GA_3	Gibberellic acid
HNMR	^1H nuclear magnetic resonance
IAA	Indole-3-acetic acid
IR	Infrared
LA	Levulinic acid
LSS	Lyophilized slimy secretion
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide phosphate, reduced form
NCS	Narciclasine
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
UV	Ultraviolet

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Chapter 1. Introduction

It has been well documented that extremely low concentrations of plant growth substances have the ability to regulate many aspects of plant growth and development from seed germination through senescence and death of the plant. More than 60 years ago auxins, which are the first class of plant growth substances, were discovered. Since then four additional classes of plant growth substances have been recognized, namely, gibberellins, cytokinins, abscisic acid, and ethylene. These five types of plant growth substances are the so-called plant hormones. Most recently brassinosteroids, salicylates, and jasmonates are beginning to gain acceptance as the newest classes of plant growth substances. Numerous advances in the use of plant growth substances on a practical scale along with basic research at the biochemical, physiological, and molecular levels have been made. At the present time plant growth substances are used in agriculture for purposes such as delaying or promoting ripening, induction of rooting, promotion of abscission, control of fruit development, weed control, size control and many other responses. Although they are currently used in agriculture, there are many questions which remain to be answered in order to maximize the true potential of plant growth substances (Arteca, 1996).

Alkaloids are structurally the most diverse class of secondary metabolites, and over 5,000 compounds are known. The term "alkaloid" is applied to nitrogen-

containing compounds, produced primarily in higher plants, but also in micro-organisms and animals, that have significant diversitied activities The chemistry of secondary metabolites is often better known than their biological activities, since phytochemical research is mainly concerned with structural aspects of them. Recently, attention of investigators has partly turned to the discovery of the biological functions of natural products during the plant life cycle and to the study of the molecular bases of these functions, as well as quantitative determination of their levels in plants (Ballio,1994).

The alkaloids are most commonly encountered in the plant kingdom, but representatives have been isolated from most other orders of organisms ranging from fungi to mammals. The studies on their isolation, structure elucidation, synthesis, and biosynthesis have resulted in thousands of publications dating from the late 1800's to the present time. The manifold pharmacological activities have always excited man's interest since early times selected plant products have been used as poisons, euphoriants or medicines etc. (Mann, 1994). Many of modern drugs now contain the same compounds or synthetic analogues, and the pharmacological and toxicological properties of these compounds are thus of immense interest and important. In plant the alkaloids are considered as growth regulators, mainly as inhibitors (Waller and Nowacki, 1978).

The Amaryllidaceae alkaloids constitute an important group of naturally occurring bases possessing a diversity of functionality and structure (Martin, 1987). Over the past 2,400 years, primitive medical records have revealed the use of extracts from many species of the plant family Amaryllidaceae for a variety of human medical problems, and cancer

treatment represents a prominent application (Pettit et al., 1993; 1995a; 1995b). Over 100 alkaloids have been isolated from plants of Amaryllidaceae with diverse biological properties (Martin, 1987). *Narcissus* is the largest genus of Amaryllidaceae. *Narcissus tazetta* is a perennial plant with an ovoid bulb grown in China. It is cultivated for ornamental purposes. Slimy substances will secret from *Narcissus* bulbs soaked in water. If cuts are introduced on the bulbs, secretion of slimy substances will increase. Some sticky fluid will secrete especially from the wound surface. The removal of slimy secretion from the cuts by washing will accelerate the sprouting and flowering of *Narcissus* (Poon, 1986). The lyophilized slimy secretion showed a wide range of bioactivities, such as inhibiting seed germination and preventing the elongation of the shoots and roots. They discovered that the inhibitory substance is soluble in ethyl acetate and 70% ethanol. Thus, a large-scale isolation and purification of this inhibitory substance from slimy mucilages of *Narcissus tazetta* bulbs was done in this study. The inhibitory substance was identified as narciclasine on the basis of its IR spectrum, UV spectrum, Mass spectrum, ^{1}HNMR spectrum and X-ray analysis. This substance had been already isolated from bulbs of *Narcissus* species (Piozzi et al., 1968) and other Amaryllidaceae plants (Piozzi et al., 1969; Pettit et al., 1986, 1993). It has been found that narciclasine is an antitumor agent which exerts an antimitotic effect during metaphase by immediately terminating protein synthesis in eukaryotic cells at the step of peptide bond formation (Jimenez et al., 1975; 1976; Mondon and Krahn, 1975; Carrasco et al., 1975), apparently by interaction with the ansiomycin area of the ribosomal peptidyl transferase center (Carrasco et al., 1975). Narciclasine has also been showed to inhibit HeLa cell growth and to stabilized HeLa cell polysomes *in vivo*, as well as to block protein synthesis in ascites

cells (Carrasco et al., 1975; Jimenez et al., 1976). Although DNA synthesis was retarded by narciclasine, RNA synthesis was practically unaffected (Carrasco et al., 1975; Jimenez et al., 1975). The recent studies show narciclasine has antiviral (RNA) activity (Gabrielsen et al., 1992), and is an antineoplastic agent (pettit et al., 1986, 1993, 1995a, 1995b). However, the biological activities of this substance in plants has so far not been reported. This thesis is to investigate the biological and physiological effects of pure narciclasine on seed germination and seedling growth, the expanding and greening of excised radish cotyledons, chlorophyll synthesis of etiolated wheat leaves, as well as electron microscopic study on excised radish cotyledons which were incubated at different conditions. The results showed that NCS had very similar action with ABA. The inhibitory activity of NCS was higher than those of ABA either in seed germination or in the growth of seedling. From the ultrastructural study on excised radish cotyledons, the degradation of lipid bodies and protein bodies, as well as the chloroplast development of excised radish cotyledons was significantly inhibited by higher concentrations (10^{-6} - 10^{-5}M) of NCS.

Chapter 2. Literature review

2.1. General information of plant growth regulators

The growth and development of plants are controlled by endogenous substances which are called plant growth regulators (plant growth substances). In the early 1900s Went (1928) made the profound statement "*Ohne Wuchstoff, kein Wachstum*", translated, "without growth substances, no growth". Plant growth regulators are organic compounds, other than nutrients. These growth regulators are frequently classified as substances which stimulate growth in physiological concentrations, i.e. stimulators, and those which inhibit growth , i.e. inhibitors. Their effect, however, is dependent not only on the concentration but also on the type of growth process, plant species, its age and physiological condition, it may shift from stimulation to inhibition, and vice versa (Machackova, 1992). In plants, there are many kinds of them. The most important of these substances are the so-called plant hormones (phytohormones). They can be defined as which (Takahashi, 1986): (1) are biologically synthesized in the plant. (2) are broadly distributed within the plant. (3) show specific biological activity in very low concentration and play a fundamental role in regulating physiological activities *in vivo*. (4) are translocated within the plant from a biosynthetic site to an action site. Typically, there is a specific, regulated mechanism for the biosynthesis of the phytohormone, as well as a regulated mechanism for destroying the phytohormone. As a result, the levels of the phytohormone in the plant are modulated. Environmental factors such

as light, photoperiod, and gravity can affect the biosynthesis, destruction and distribution of plant hormones and, in turn, the hormones modify the developmental program or the growth response.

There are currently only five generally acknowledged groups of phytohormones, even though more will almost surely be discovered. The five groups of phytohormones include auxins, cytokinins, gibberellins, abscisic acid and ethylene. Each of the five major phytohormones has been implicated in regulatory control of specific processes in the plant at the cellular, tissue , or plant level; with time, nearly all of these specific functions have proved to be very complex. Auxin was the first plant hormone to be identified. In the mid-1930s two groups of researchers, one in the Netherlands and the other in the United States, discovered the chemical nature of auxin. Work done primarily by Kögl et al. (1934) in Holland and by Thimann (1935) in the United States led to the discovery that auxin is indole-3-acetic acid (IAA). It is the most common form of auxin in the plant kingdom. Auxin has a wide variety of effects on plant growth and development. Auxin is well known for its strong effect in stimulating elongation in isolated stem segments (Cleland, 1995), implying a possible role for endogenous auxin in the control of stem elongation in intact plants (Yang et al., 1996). A close correlation between the endogenous levels of IAA and stem growth exist in a range of genetic lines of peas differing in height (Law and Davies, 1990). It has been demonstrated that intact light-grown pea plants are capable of a sustainable, strong response in stem elongation to exogenously applied IAA, provided the application is continuous (Behringer et al., 1992; Yang et al., 1993).

The gibberellins were first discovered by Kurosawa (1926) in Japan in the course of his studies on a fungal disease in rice. He was investigating the mechanism by which the fungal pathogen, *Gibberella fujikuroi*, brought about the abnormally elongated stems that characterize seedlings infected with this pathogen. Kurosawa showed that this abnormal elongation was due to a water-soluble compound produced by the fungus which he called gibberellin A. Later, gibberellin A was shown to be a mixture of six different gibberellins and found to be in higher plants as well as some fungi. Gibberellins are a family of compounds including seventy-nine free gibberellins and more than 10 gibberellins conjugates have been chemically characterized as naturally occurring (Takahashi, 1991). The basic plant physiological research has been intensively conducted, and much notable progress has been achieved. The scope of research has extended to the molecular mechanism of the action of gibberellins, and investigations on the purification of the enzyme participating in the biosynthesis of gibberellins and on the genetic background of the enzyme are in progress. Gibberellins are known to regulate gene expression in the aleurone layer of germinating cereal seeds (Hooley, 1994), in vegetative shoot tissue (Chory et al., 1987; Shi et al., 1992) and flowers (Weiss et al., 1990; Jacobsen et al., 1994). Although most studies of GA-regulated genes focus on genes whose mRNAs are increased in abundance, studies of barley or wheat aleurone layers have also found RNAs which decrease in abundance following GA treatment with a relatively late time course (Baulcombe and Buffard, 1983; Nolan and Ho, 1988; Heck et al., 1993). In barley aleurone

layer, three RNA species were found to decrease in abundance following treatment with Gibberellic acid (GA_3) (Nolan and Ho, 1988; Heck et al., 1993).

Cytokinins were discovered as a result of efforts to find factors that would stimulate plant cells to divide (Sovonick-Dunford, 1991). This work that led to the isolation and identification of the first substance which promoted plant cell division was derived directly from the studies of Skoog and his co-workers on plant tissue cultures. Jablonski and Skoog (1954) found that tobacco pith tissue responded by an enormous cell enlargement entirely unaccompanied by cell division in the presence of suitable concentration of auxin in a synthetic basal medium. Cell division did occur, however, in pith tissue with attached vascular strands, and in severed pith tissue placed in contact with vascular tissue. The material active in inducing cell division was found by these workers to be contained in extracts of the vascular tissue, coconut milk or malt extract. A potent cell division promoting activity was later found in aged DNA or autoclaved DNA under acidic conditions. In 1955, Miller et al. isolated a highly active compound from a rich source of autoclaved herring sperm DNA and identified it as 6-furfurylaminopurine. It was given the name kinetin. Virtually all the known naturally-occurring cytokinins are substituted purines (Machackova, 1992). Although cytokinins were defined as cell-division factors (Miller et al., 1955; Skoog et al., 1965), they influence a wide array of important biological processes. Numerous investigations showed that cytokinins play a role, major or minor, throughout development, from seed germination to leaf and plant senescence, and modulate physiological processes important throughout the life of a plant, including photosynthesis

and respiration (Mok, 1994). Experimental evidence indicates that is a close association between the regulation of gene expression and cytokinin action (Chen and Leisner, 1985; Flores and Tobin, 1988; Cotton et al., 1990; Lu et al., 1990, 1992; Dominov et al., 1992; Sugiharto et al., 1992; Chen, et al., 1993; Teramoto et al., 1993; Binns, 1994; Crowell, 1994; Ye and Varner, 1994).

Abscisic acid (ABA) was discovered by two research groups almost at the same time. Addicott's (1964) group was searching for factors causing the abscission of fruits and leaves, and Wareing's (1964) group was studying the regulatory signals controlling the onset of dormancy. Abscisic acid was discovered as a growth inhibitor that plays a major role in seed and bud dormancy. In addition, ABA also regulates other several important aspects of plant growth and development, such as the control of stomatal closing under water stress, initiation of senescence, etc. Research on the mechanism of grain seeds dormancy suggests strong involvement of ABA (Robichaud et al., 1980; Fong et al., 1983; Karssen et al., 1983; Koornneef et al., 1984; Kermode, 1990). Both ABA content and ABA sensitivity influence seed dormancy and germination (Robertson et al., 1980; Fong et al., 1983). Addition of ABA inhibits germination of embryo isolated from various plant species (Robertson et al., 1989; Reid and Walker-Simmons, 1990; Corbineau et al., 1991). Embryos isolated from dormant grains are more sensitive to exogenous ABA-induced inhibition of germination than embryos isolated from non-dormant grains (Walker-Simmons, 1987; Reid and Walker-Simmons, 1990; Van Beckum et al., 1993, Wang et al., 1994). From the measurement of Wang et al. (1995), they propose that the inability of dormant grains to

germinate is due to a high amount of endogenous ABA in the embryos and to the high ABA sensitivity of the embryo, which together inhibit the growth of the embryo. Although nondormant grains also have a considerable amount of ABA, their low ABA sensitivity permits germination. So intact grains will germinate when either the ABA sensitivity is low (nondormant condition) or when ABA is able to diffuse out of the embryo (dormant condition). The diffusion of ABA out of the embryo may occur from the embryo to the rest of grain or to the outside (Wang et al., 1995).

Ethylene is a simple molecule. It is the only known gaseous plant hormone. The first indication that ethylene is a natural product of plant tissues was reported by Cousins (1910) who noticed that when oranges were packed and shipped in the same container with bananas, the bananas ripened prematurely. In 1934, Gane identified ethylene as a natural product of plant metabolism, and because of its effects on the plant growth and development , it was later classified as a hormone. Ethylene production is highly regulated during plant development, being induced during seed germination, ripening of fruits, abscission of leaves, and senescence of flowers. A variety of extrinsic factors including mechanical wounding, various environmental factors, and certain chemicals also induce ethylene production (Matto and White, 1991). There has been increasing evidence to suggest that many of the response of plants to ethylene involve changes in the pattern of gene expression (Christoffersen and Laties, 1982; Zurfluh and Guilfoyle, 1985; Schuch et al., 1989). A role for ethylene translocation in the senescence in some flowers was suggested by Woltering (1990), O'Neill et al. (1993) and Woltering et al. (1995).

On the other hand, the different types of plant hormones can act synergistically or antagonistically (Thimann, 1992), for instance, cytokinins delay leaf senescence, but so do auxins and gibberellins, while abscisic acid and ethylene promote senescence (Mok, 1994). Auxins alone may not account for cell enlargement, and gibberellin alone does not control stem growth (Cleland, 1964). Yang et al. (1996) suggest that auxin is an essential factor affecting stem elongation in addition to gibberellin and that the two hormones may control separate processes that together contribute to stem elongation. Cytokinin alone does not appear to induce cell division even in the standard cell division tests, the assay for cytokinins requires the presence of auxin (Skoog and Miller, 1957; Steward and Shantz, 1959). Abscisic acid itself does not adequately account for the control of either abscission (Craker and Abeles, 1969; Nooden and Leopold, 1978) or dormancy (Lenton et al., 1972; Loveys et al., 1974; Nooden and Weber, 1978; Phillips et al., 1980). Exogenous gibberellic acid (GA_3) application has proved to be effective in breaking dormancy and in substituting the requirement of cold stratification in many seeds (Powell, 1987). Moreover, GA_3 stimulates germination and reverses the effects of ABA in these and other processes, antagonistically affecting the synthesis of specific mRNAs and proteins (Jacobsen and Beach, 1985; Rodriguez et al., 1987). The findings obtained by Nicolas et al. (1996) suggest that both ABA and GA_3 could be involved in the regulation of nucleic acid and protein metabolism during dormancy, acting antagonistically in these processes and, specifically, in the regulation of the two proteins that appear to play a role in the maintenance of dormancy in *Fagus sylvatica* seeds.

Apart from these important plant growth regulators-phytohormones, there are many other plant growth regulators that show interesting physiological activity in plants. In general these substances are of limited distribution in the plant kingdom and show a rather restricted range of activities. Of these regulators, some are the stimulators (Fig. 2.1), and the others are inhibitors (Fig. 2.2) (Takahashi, 1986).

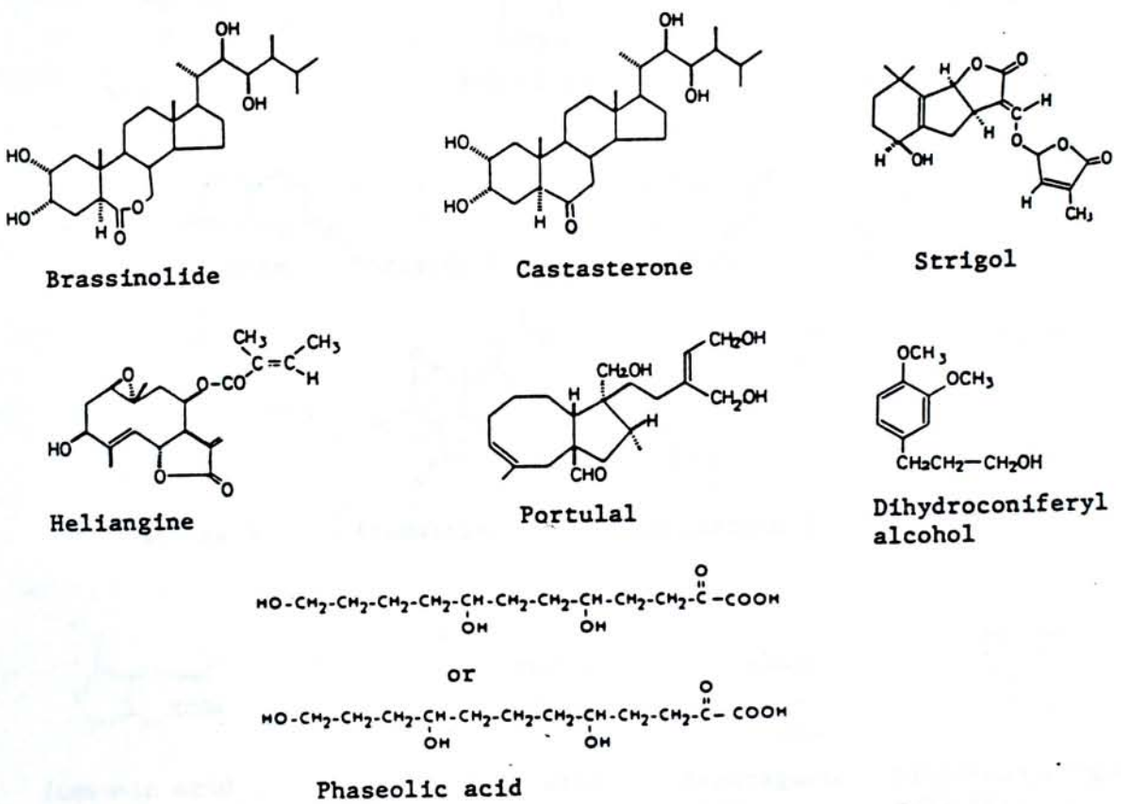


Fig. 2.1 Structures of plant growth promotors

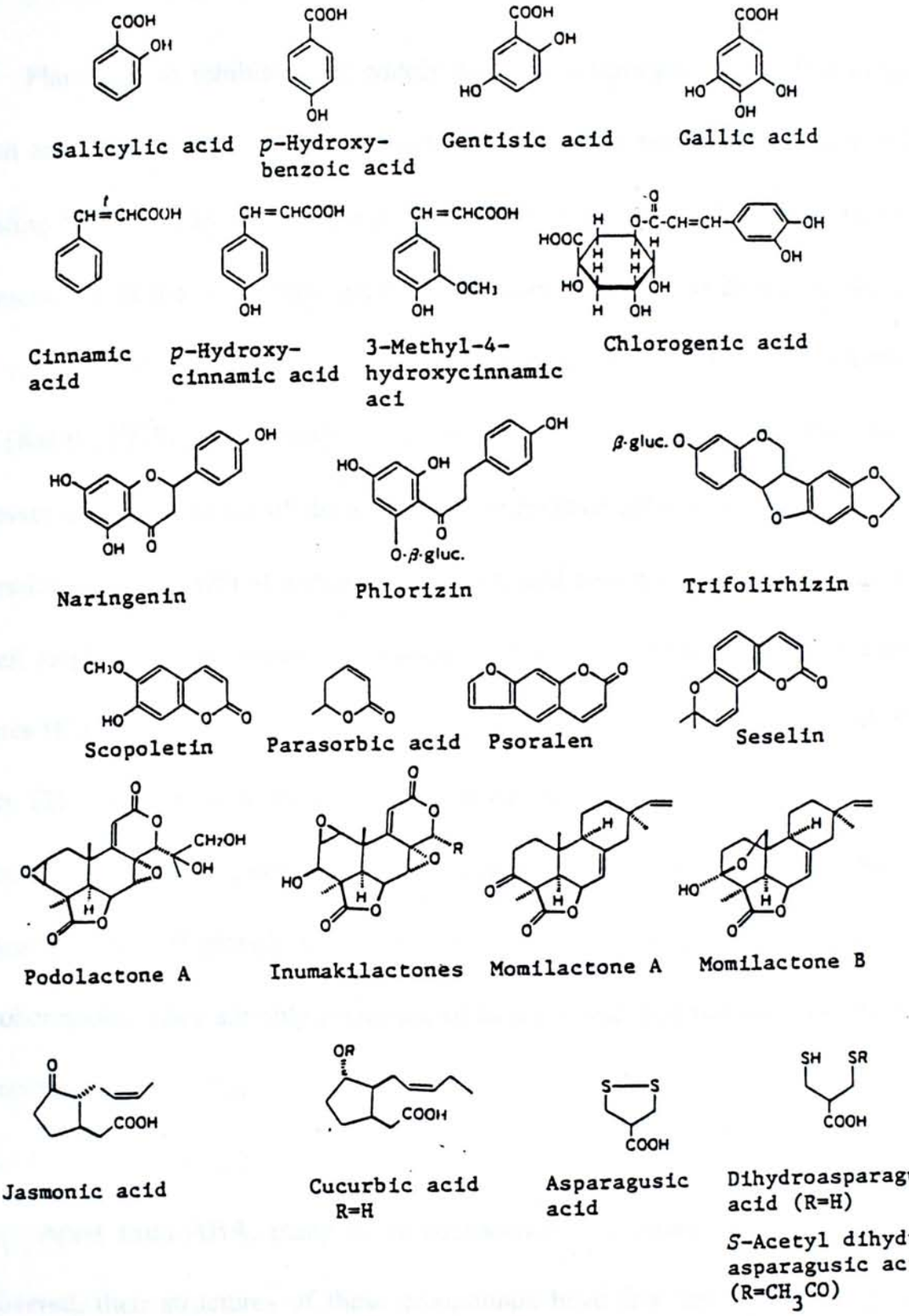


Fig. 2.2 Structures of some plant growth inhibitors

2.2. Natural plant growth inhibitors

Plant growth inhibitors are widely distributed throughout the plant kingdom. Plant growth and development can be interpreted in terms of a balance between stimulating and inhibiting factors. They play a role in the control of growth and development of plants in conjunction with the other plant growth regulators (substances) including phytohormones. They are found not only in dormant organs, but also in growing part -- leaves, stems and roots (Kefeli, 1978). Plant growth inhibitors have been shown to participate activity in the processes at the inception of dormancy, in correlative growth inhibition, leaf abscission, depression of the growth of a stem and its part, and also in inhibition of seed germination, as well as phytohormones-induced processes. Plant growth inhibitors have some common features (Kefeli and Kadyrov, 1971): (1) Their levels vary during the period of growth of plants. (2) They are able to inhibit growth of different isolated organs or tissues of the plants. (3) They do not possess specific antihormonal activity. (4) They are not able to depress all forms of growth processes. (5) They are present in young green tissues with phytohormones. They are only accumulated in the absence of phytohormones, in senescent and resting organs.

Apart from ABA, many other compounds that usually inhibit growth have been discovered, their structures of these compounds have few similarities, they are capable, under certain circumstances, of altering the course of plant development, such as tricontanol (Laughlin et al., 1983), polyamines (Galston, 1983), brassinosteroids (Cohen and Meudt,

1983), batatasin (Hasegawa and Hashimoto, 1975), pisumin (Hasegawa et al., 1983), *Eucalyptus* growth regulators G (Panton et al., 1980), jasmonic acid (Yamane et al., 1981) etc.

2.3. Alkaloids and narciclasine

In addition to the above mentioned endogenous plant products, others are products of plants or microorganisms, the so-called secondary metabolites, that have been more or less unexpectedly found to affect plant growth. This group consists of a vast set of compound with various chemical natures, often their chemistry is better known than their biological activities. For a long time, phytochemical research has been mainly concerned with structural aspects of secondary metabolites. However, in recent years attention has partly turned to the discovery of the biological functions of natural products during the plant life cycle and to the investigation of the molecular bases of these functions, as well as changes of these compounds (Ballio, 1994).

The alkaloids are one of the largest and most diverse families of secondary metabolites and contain some of the most complicated molecular structures, and over 5,000 compounds are known. They are most commonly encountered in the plant kingdom, but representatives have been isolated from most other orders of organisms ranging from fungi to mammals. The term "alkaloid" is applied to nitrogen-containing compounds. And nitrogen atom in these substances is usually part of a heterocyclic ring , a ring containing

both nitrogen and carbon atoms. Studies on their isolation, structure elucidation, synthesis, and biosynthesis have resulted in thousands of publications dating from the late 1800's to the present time. A large percentage of these investigations were stimulated by native folklore medicine from which claims about medicinal qualities of appropriate, plant extracts eventually led to the realization that the active principle was an alkaloidal component (Kutney, 1987). Manifold pharmacological activities of alkaloids have always excited man's interest, and since early times selected plant products (many containing alkaloids) have been used as poisons for hunting, murder and euthanasia; as euphoriants, psychedelics, and stimulants; or as medicines. Many of our modern drugs now contain the same compounds or synthetic analogues, and the pharmacological and toxicological properties of these compounds are thus of immense interest and importance (Mann, 1994).

The role of alkaloids in plants has been a subject of speculation for at least 100 years. Alkaloids were once thought to be nitrogenous wastes (analogous to urea and uric acid in animals), nitrogen storage compounds, or growth regulators, but there is little evidence to support any of these functions (Lorimer, 1991). The alkaloids are considered as growth regulators in plant, mainly as inhibitors, since the structures of some of them resemble structures of known growth regulators (Waller and Nowacki, 1978). The best known example of an alkaloid which inhibits cell division in plants is colchicine. Added in minute amounts, this alkaloid interferes with the formation of the cell carokinetic spindle; instead of a division of the cell into two daughter cells, a restitutive cell is formed with a doubled set of chromosomes. Alkaloids of *Senecio* and *Crotalaria* can cause chromosome

breakage in a number of organisms, mostly animals, but are harmless to the species producing them. Some alkaloids, when added to water in which seeds of alkaloid-free plants are soaked, inhibit germination. Some alkaloids prevent the germination of seeds of foreign species, thus preserving space for its own progeny. However, it was found that some plants can absorb alkaloids from the soil without serious effects. In cases where it has been proved that an alkaloid is poisonous to a foreign plant, a postulation can be made that the alkaloid-producing species has evolved a system that can effectively operate in spite of the present of alkaloid. Table 2.1 shows some examples of the known biochemical effects of alkaloids. It is important to recognize that these processes do not occur in all plants. An alkaloid common for a plant family or a similar taxonomical unit most probably originated together with the group of the species (Waller and Nowacki, 1978).

Amaryllidaceae plants were recorded in the literature as well established treatments for cancer (Cook and Loudon, 1952). The Amaryllidaceae alkaloids constitute an important group of naturally occurring bases possessing a diversity of functional and structure (Martin, 1987). Over 150 alkaloids have isolated from plants of the Amaryllidaceae with diverse biological properties (Martin, 1987). At present, eleven fundamental ring systems have been known. Representative alkaloids from each of these classes include lycorine, lycorenine, narciclasine, galanthamine, crinine, vittatine, pretazettine, latisodine, cherylline, montanine and amisine, respectively (Sener et al., 1992). Some alkaloids of the Amaryllidaceae have attracted great attention as a consequence of their biological activities. For example, pretazettine has been involved in a number of interesting activities including

Table 2.1 Some Other Biochemical Effects of Alkaloids in Plants[a]

Alkaloid tested	Primary action	Later effect	System used	Reference
Theophylline	Inhibits 3',5'-phosphodiesterase	α-Amylase released	Barley endosperm	Duffus & Duffus (1969)
Quinine	Intercalates in DNA helix	Phenylalanine-ammonia lyase induced	Pea pods	Hadweiger & Swochaw (1971)
Caffeine	Binds to part of operon	Adenylsuccinate lyase activity increased	*Bacillus subtilis*	Nishikawa & Shiio(1969)
Diaminosteroid alkaloids	Complexes with DNA	Replication inhibited	Bacteriophage	Mahler & Baylor (1967)
Colchicine	Cell carokinesis	Cell is formed with a double set of chromosomes	Most plants	Eigsti & Dustin (1955)
Colchicine	Binds the purified microtubular protein	In chromosome movement during mitosis, etc.	Cells of a general type	Sherline et al. (1975); Olmsted& Borisy(1973)
Veratrum alkaloids	Inhibits growth	Effect on DNA stability	Rye, oats	Olney (1968)
Nicotine	Inhibits chlorophyll synthesis	-	-	Hassall (1969)
Gramine	Plant competition	-	Barley	Overland (1966)
Lycorine	Inhibits growth of root	-	-	Ghoshal et al. (1984)
Lycorine	Inhibits biosynthesis of ascorbic acid	-	Pea seedlings, Potato slices, *Clivia* leaves	Arrigoni (1975)
Lycorine	Prevents the elicitation of anticyanid respiration	-	Potato slices	Arrigoni et al. (1976)
Raphanusanin	Inhibits hypocotyl growth	-	Etiolated radish and lettuce seedlings	Hasegawa et al. (1982); Sakoda et al. (1991)
Tryptophan	Inhibits root elongation	-	Oat seedlings	Haeggquist et al. (1988a, 1988b)

[a]Patterned after Robinson (1974); Waller and Nowacki (1978).

inhibition of protein synthesis in eucaryotic cells by interfering with the peptide bond formation step, inhibition of purified RNA-dependent DNA polymerase from avian myeloblastosis virus and therapeutical efficacy against the *Rauscher leukemia* virus and carcinomas (Furusawa et al., 1981). Some of them have been used in the treatment of myastenia gravis, myopathy and the diseases of the nervous system (Ali et al., 1986). Galanthamine, a widespread alkaloid among the Amaryllidaceae plants exhibits an analgesic activity comparable to that of morphine. It has also shown to exhibit reversibly cholinesterases and it has been found to possess insecticidal activity (Numata et al., 1983). Lycorine is also the most widely distributed alkaloid amongst the Amaryllidaceae plants, and it shows several biological effects. This alkaloid displays antiviral properties and was shown to inhibit ascorbic acid biosynthesis (Ghoshal et al., 1988), to cause bradycardia and to exhibit potent insecticidal action against the yellow butterfly (Martin, 1987). In addition, lycorine also shows emetic properties. The hydrogenation of the double bond of lycorine forms dihydrolycorine in which the emetic causes an antiarrhythmic effect. Besides, lycorine has been shown to possess plant-growth inhibiting property (Martin, 1987).

Narcissus is the largest genus of Amaryllidaceae (Sener et al., 1992). The medicinal properties of extracts from *Narcissus poeticus L.* were noted by the Greek physician Hippocrates Cos as early as 300 B.C. and by the Romans in the first century A.D (Gabrielsen et al., 1992). Over the past two decades, a number of potentially important constituents of this family have been identified as I socarbostyrils, such as narciclasine

whose extraction and antimitotic properties have been described by Ceriotti (1967), lycoricidine (7-deoxynarciclasine), pancratistatin (Pettit et al., 1984a, 1984b, 1986, 1993, 1995a, 1995b) and the alkaloids lycorine, pseudolycorine and pretazettine (Gabrielsen et al., 1992). Pancratistatin and 7-deoxynarciclasine have been isolated from the bulbs of Hawaiian *Pancratium littorale* Jacq. and *Zepbyrantbes grandiflora* and their structures elucidated (Pettit et al., 1986). Considerable efforts (Danishefsky and Lee, 1989; Thompson and Kallmerten, 1990) have recently culminated in the total synthesis of racemic pancratistatin by Danishefsky and Lee (1989). *Trans*-Dihydronarciclasine, the principal antiviral and cytostatic constituent of the Chinese medicinal plant *Zepbyrantbes candida*, has also been recently isolated (Pettit et al., 1990). Lately with regard as the biological properties of them, lycorine was found to be responsible for the antiviral activity of leaf and root extracts of the Amaryllidaceae plant *Clivia miniata* Regel against Herpes simplex, Semliki forest, polio, Coxsackie, and measles viruses in Vero cells (Ieven et al., 1982). Such biosynthetic products exert their biological activities mainly by inhibiting protein synthesis in peptide bond formation. Study of the effect of lycorine on viral protein formation in poliovirus-infected HeLa cells serves as a useful illustration (Vrijsen et al., 1986). Pseudolycorine and pretazettine similarly inhibit protein synthesis and have manifested activity against murine Rauscher leukemia virus and neurotropic RNA viruses (Zee-Cheng et al., 1978). Some reports (Papas et al., 1973) describe the antiviral properties of crude extracts containing the isocarbostyril-type compounds against neurotropic RNA viral infections in mice with Japanese encephalitis virus and lymphocytic choriomeningitis

(LCM) virus (Furusawa et al., 1971; Kinstle et al., 1966). In 1992 (Gabrielsen et al.), a series of 23 Amaryllideceae isoquinoline alkaloids and related synthetic analogues were isolated or synthesized and subsequently evaluated in cell culture against the RNA-containing flaviviruses (Japanese encephalitis, yellow fever, and dengue viruses), bunyaviruses (Punta Toro, sandfly fever, and Rift Valley fever viruses), alphavirus (Venezuelan equine encephalomyelitis virus), lentivirus (human immunodeficiency virus-type 1) and the DNA-containing vaccinia virus. Narciclasine, lycoricidine, pancractistatin, 7-deoxypancratistatin, and acetates, isonarciclasine, *cis*-dihydronarciclasine, *trans*-dihydronarciclasine, their 7-deoxy analogues, lycorines and pretazettine exhibited consistent *in vitro* activity against all three flaviviruses and against the bunyaviruses, Punta Toro and Rift Valley fever virus. Activity against sandfly fever virus was only observed with 7-deoxy analogues (Gabrielsen et al., 1992).

Pettit and his colleagues (1993) found that the bulbs of *Hymenocallis littoralis* and *Hymenocallis caribaea*, as well as *Hymenocallis latifolia* contain a cytotoxic, isocarbostyril -type biosynthetic product, 7-deoxy-*trans*-dihydronarciclasine. This new compound inhibited the cytopathicity and/or replication of various viruses. Companion cytotoxic constituents of *Hymenocallis littoralis* and *Hymenocallis caribaea* were found to be pancratistin, narciclasine and 7-deoxynarciclasine. Although there were striking differences in overall potency, some of the compounds shared a highly characteristic differential cytotoxicity profile against the 60 diverse human tumor cell lines comprising the NCI panel. As a group, the melanoma subpanel lines were most sensitive; certain individual lines

within other subpanels (eg., NSC lung, colon, brain, renal) were as much as a thousand-fold or more sensitive than the less sensitive lines (Pettit et al., 1993).

Narciclasine, one kind of alkaloid, had been isolated from *Narcissus* bulb and other Amaryllidaceae bulb (Piozzi et al., 1968; Piozzi and Marino, 1969; Pettit et al., 1986, 1993). It has been found that narciclasine exhibits a wide range of biological activities, especially in medicine. The antitumor activity of crude preparations of bulbs from species of *Narcissus* (Fitzgeoald et al., 1958) is due to narciclasine (Piozzi et al., 1968; Fuganti et al., 1971), which exerts an antimitotic effect during metaphase. This can be explained by the strong inhibitory effect of narciclasine on protein synthesis in eukaryotic ribosomes, which is due to its interaction with the peptidyl transferase center of the larger subunit of eukaryotic ribosomes (Carrasco et al., 1975; Jimenez et al., 1975). This mode of action apparently results from an inhibitory effect of narciclasine on protein synthesis since the compound blocks translation in Ehrlich as cites tumor cells and also as stabilizes polyribosomes in HeLa cell (Jimenez et al., 1976). Recently, it has been indicated that narciclasine exhibits the antiviral (RNA) activity (Gabrielsen et al., 1992), and this compound is also an antineoplastic agent (Pettit et al., 1986, 1993, 1995a, 1995b).

2.4. Studies on expansion and greening of cotyledons

The cotyledons of several plant species undergo drastic changes in function and in cell structure during germination in light. Reserve materials gradually disappeared and the

storage function is substituted by the photosynthetic function (Kagawa et al., 1973). Plastids and cell organelles (glyoxysomes and peroxisomes) are deeply involved in these transformations. Plastids develop from proplastids (that may contain storage material) to normal chloroplasts. Glyoxysomes increase during the first few days of germination and decline when most of the lipid reserves have been used up. The decline of glyoxysomes coincides with a strong increase in the level of peroxisomal enzymes (Longo et al., 1979).

Exogenous application of cytokinins promotes the expansion of cotyledons and accelerates their development from storage organs to photosynthetic organs (Longo et al., 1979). Cytokinins-induced expansion of excised cotyledons of various species represents a rapid and convenient bioassay for such hormones (Letham, 1971; Narain and Laloraya, 1974). The mechanism of this response has been reported by Gordon and Letham (1975); Huff and Ross (1975); Bewli and Witham (1976); Rijven (1976); and Logno et al. (1978). These studies indicate that although cell division is promoted slightly, the major cause of expansion of excised cotyledons is the increased cell expansion resulting from water absorption.

Concerning cotyledons of cucumber (Harvey et al., 1974), sunflower (Servettaz et al., 1976), and watermelon (Logno et al., 1978), evidence suggests that cytokinins promote changes from a storage to a photosynthetic function. Cytokinins stimulate conversion of proplastids to chloroplasts in cotyledons or other tissues (Stelter and Laetsch, 1965; Harvey et al., 1974; Wozny and Szweykowska, 1975; Farineau et al., 1978). Enhanced chloroplast

development in cotyledons is sometimes accompanied by more rapid disappearance of storage compounds. Accelerated disappearance of protein bodies in flax (Sveshnikova and Khokhlova, 1968) and of both protein bodies and lipid bodies in cucumber (Wozny and Szweykowska, 1975) and watermelon (Logno et al., 1978, 1979) is observed after cytokinin treatment. It is likely that the glyoxylate pathway is accelerated by cytokinins, because they increase the activity of isocitrate lyase in squash (Penner and Ashton, 1967), sunflower (Servettaz et al., 1976) and watermelon (Longo et al., 1978).

There are two major functional classes of cell organelles in higher plants: glyoxysomes and peroxisomes. These two classes of cell organelles have been the subject of many studies (Tolber, 1971, 1981; Beevers, 1979; Kindl and Lazarow, 1982; Huang et al., 1983; Lord and Roberts, 1983; Trelease, 1984; Lazarow and Fujiki, 1985; Borst, 1989; Olsen and Harada, 1991, 1995; Kindl, 1992; Van den Bosch, 1992; Subranmani, 1993; Olsen and Harada, 1995). Glyoxysomes are found in fat-storing cells of seeds during germination. Glyoxysomes possess all of the glyoxylate-cycle enzymes and the β-oxidation enzymes (Breidenbach et al., 1967; Cooper and Beevers, 1969; Hutton and Stumpt, 1969). These enzymes catalyze the net conversion of fatty acids to succinate and are largely responsible for the ability of plants to utilize lipids as a carbon source (Beevers, 1980; Trelease, 1984). Succinate generated by these reactions is generally converted to carbohydrates by gluconogenic enzymes in the cytosol and mitochondria. Glyoxysomes have been characterized most extensively during the postgerminative development of oilseed plants, where they are involved in mobilizing storage lipids to

provide nutrients for growing seedlings. Functional glyoxysomes also occur in senescent organs, presumably in response to the mobilization of membrane lipids (Gut and Matile, 1988; Birkhan and Kindl, 1990; De Bellis et al., 1990, 1991). Additionally, the activities of two enzymes, isocitrate lyase and malate synthase, associated exclusively with glyoxysomes have been detected in developing seeds (Comai et al., 1989; Ettinger and Harada, 1990; Turley and Trelease, 1990) and pollen (Zhang et al., 1994), but it is unclear whether the other glyoxylate-cycle enzymes are also present at these stages of the life cycle.

Isocitrate lyase and malate synthetase are the key enzymes of the glyoxylate pathway (Carpenter and Beevers, 1959; Yamamoto and Beevers, 1960; Presley and Fowden, 1965; Mori and Nashimura, 1989), and hydroxypyruvate reductase and glycolate oxidase are the key enzymes of the peroxisomes (McGregor 1969; Tolbert et al., 1970; Kagawa et al., 1973; Kagawa and Beevers, 1975; Becker et al., 1978). Studies with a variety of species have established a characteristic developmental pattern for the key enzymes of the glyoxylate cycle during the early postgerminative growth of light-grown fatty seedlings. Little or no isocitrate lyase or malate synthetase is detected in dry seeds (Marcus and Velasco, 1960), but both enzymes increase markedly in activity shortly after germination, reach a peak within a few days, and then decline rapidly as lipid reserves are depleted (Carpenter and Beevers, 1959; Ching, 1970; Firenzuoli et al., 1968; Karow and Mohr, 1967; Lado et al., 1968; Lee et al., 1964; Longo and Longo, 1970; Marcus and Velasco, 1960; McGregor and Beevers, 1969; Trelease et al., 1970a,

b). The postgerminative increase in activity is known to result from *de novo* enzyme synthesis both in endosperm (Lado et al., 1968) and in cotyledons (Gientka and Cherry, 1968; Hock and Beevers, 1966; Longo, 1968). The cell organelles of some fatty cotyledons, however, are of further developmental interest, since the cotyledons growing in light expand and differentiate into photosynthetic organs after the depletion of lipid reserves. In such cotyledons, the activities of glyoxysomal enzymes decrease while enzyme activities of leaf peroxisomes increase correspondingly (Gruber et al., 1970; Schnarrenberger et al., 1971; Gerhardt, 1973; Kagawa et al., 1973; Drumm and Schopfer, 1974; Kagawa and Beevers, 1975). McLaughlin and Smith (1994) demonstrated that the induction of malate synthase and isocitrate lyase - glyoxysome-specific enzymes - appears to be coupled with a decline in the steady-state levels of transcripts encoding peroxisomal hydroxypyruvate reductase and the amounts of the photorespiratory enzyme serine:glyoxylate aminotransferase. It has been proposed that malate synthase and isocitrate lyase are synthesised in response to lipid degradation in detached and senescent tissue (Gut and Matile, 1988). However, McLaughlin and Smith (1994) found that malate synthase and isocitrate lyase synthesis in detached cucumber cotyledons begins before a decline in lipid can be detected. Furthermore, the activation of malate synthase and isocitrate lyase genes expression does not correlate with loss of chlorophyll, carotenoid, protein or RNA. These results are discussed in terms of genes encoding glyoxylate-cycle enzymes and their possible function in detached and senescing organs (McLaughlin and Smith, 1994).

Hydroxypyruvate reductase is one of the key enzymes of peroxisomes. It catalyzes the reduction of hydroxypyruvate to glycerate with the simultaneous oxidation of NADH to NAD^+. This reaction is part of the photorespiratory pathway and is localized in leaf peroxisomes (Tolbert et al., 1970; Titus and Becker, 1985). The appearance of hydroxypyruvate reductase in the cotyledons of cucumber seedlings is both developmentally and light-regulated (Hondred et al., 1987). Transcripts for hydroxypyruvate reductase are present in cotyledons and leaves but have not been detected in roots (Greenler et al., 1990). Recently, Andersen and his colleagues (1996) reported the isolation and sequencing of a partial hydroxypyruvate reductase cDNA from pumpkin, as well as the enhancement of both hydroxypyruvate reductase transcript levels and the rate of transcription by BA in etiolated pumpkin cotyledons. They suggest that the enhancement of this enzyme mRNA by BA is , at least in part, at the level of transcription.

Immunocytochemical analysis revealed that glyoxysomes are transformed directly into leaf peroxisomes in greening cotyledons (Nishimura et al., 1986; Titus and Becker, 1985; Sautter, 1986) and leaf peroxisomes are also converted directly to glyoxysomes in senescing cotyledons (Nishimura et al., 1993). Using cDNA for two glyoxysomal enzymes, malate synthase and citrate synthase, it has been found that decreases in activities of glyoxysomal enzymes are caused not only by decreases in the levels of corresponding mRNAs (Mori et al., 1991; Kato et al., 1995), but also by the degradation of glyoxysomal enzymes in microbodies (Mori and Nishimura, 1989). Moreover, the

decreases in activities of glyoxysomal enzymes during the microbody transition are accompanied by the appearance and increases in levels of leaf-peroxisomal enzymes, such as glycolate oxidase and hydroxypyruvate reductase. An analysis using the cDNA for a leaf-peroxisomal enzyme, glycolate oxidase, revealed that increases in the activity of glycolate oxidase are due to increase in the level of the corresponding mRNA (Tsugeki et al., 1993). These results demonstrate clearly that the matrix enzymes of glyoxysomes are replaced by those of leaf peroxisomes during the microbody transition. Yamagushi et al. (1995) reported that membrane proteins in glyoxysomes change dramatically during the microbody transition, as do the enzymes in the matrix.

2.5. Investigation on chlorophyll synthesis

Tetrapyrrole compounds are essential components of life. They include hemes, which serve as prosthetic groups of respiratory enzymes, and chlorophylls, which are the major photosynthetic light-harvesting pigments (Senge, 1993). All carbon and nitrogen atoms of tetrapyrroles are derived from δ-aminolevulinic acid (ALA) whose formation provides the basic control point in the multistep pathway of tetrapyrrole biosynthesis, especially in the case of chlorophyll during dark/light transitions (Beale and Weinstein, 1990). It has been indicated that chlorophyll synthesis involves multienzymes and multisteps (Granick, 1967). Exogenous addition of ALA to dark-grown seedlings leads to the accumulation of protochlorophyllide (Sisler and Klein, 1963; Nadler and Granick, 1970; Castelfranco et al., 1974), suggesting that all of the enzymes required for the

synthesis of protochlorophyllide from ALA are present in significant quantities in etiolated tissue and that ALA formation is the rate-limiting step in chlorophyll biosynthesis in dark-grown plants. When etiolated seedlings are first illuminated, the initial photoreduction of protochlorophyllide to chlorophyllide is not immediately followed by maximal chlorophyll synthesis. Instead, there is a lag phase during which the enzyme system responsible for ALA synthesis appears to be formed *de novo*. Indeed, after the lag phase, the rate of chlorophyll synthesis is enhanced and accompanied by a commensurate increase in level of ALA-forming activity (Castelfranco et al., 1974). In plants, algae, and certain eubacteria, including *Escherichia coli*, ALA is formed via the C_5 pathway (Beale and Weinstein, 1991; Jahn et al., 1992), as shown in Figure 2.3 (Ilag et al., 1994). The initial metabolite is the normal Glu-tRNA^{Glu} that is converted by the action of a unique enzyme, Glu-tRNA reductase (GluTR), to glutamate 1-semialdehyde (GSA) with the concomitant release of tRNA^{Glu}. GSA is then converted to ALA by a specific transaminase, GSA-2,1-aminomutase (GSA-AM). Thus, GSA is the first committed precursor of porphyrin synthesis in organelles and organisms that use the C_5 pathway. In plants, tRNA^{Glu} is chloroplast encoded, whereas the genes for GluTR and GSA-AM are believed to be nuclear encoded (Jahn et al., 1992). Glu-tRNA has a dual function: it provides glutamate for protein biosynthesis and GSA for porphyrin synthesis (Jahn et al., 1992).

Levulinic acid (LA), a structural analogue of ALA, inhibits the first enzyme in the chlorophyll biosynthetic pathway, ALA dehydratase, which condenses two molecules of

ALA to porphobilinogen (Klein et al., 1975). Application of LA to a developed green plant will not have much impact as its photosynthetic apparatus is already well developed. The rate of chlorophyll synthesis in a mature plant is also significantly lower than that of a developing and greening seedling. Therefore, the studies chlorophyll synthesis or chloroplast development by exposing etiolated plant materials to light in the presence of the LA are needed (Jilani et al., 1996).

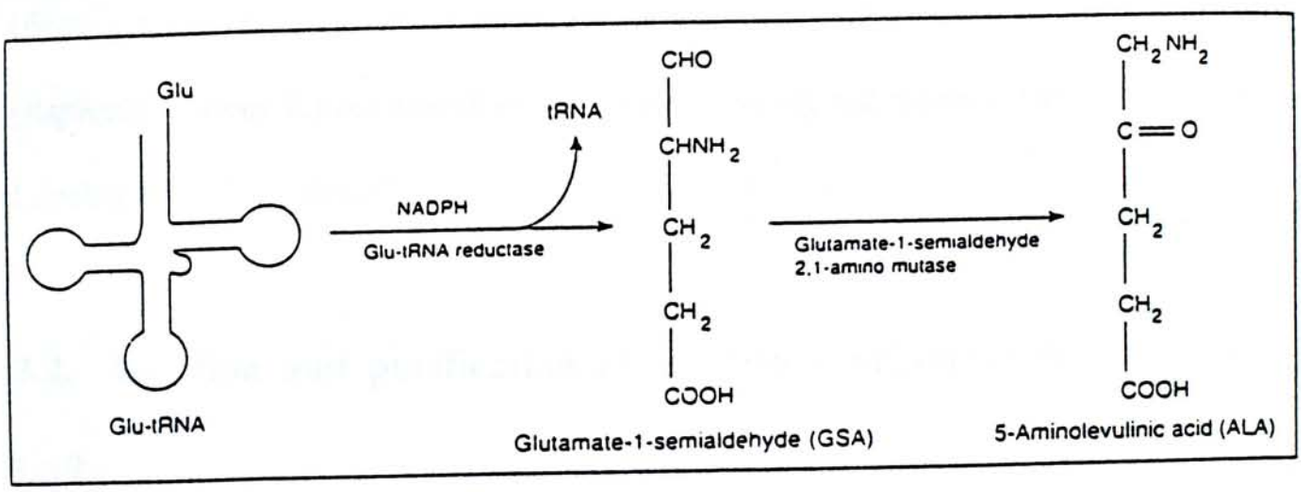


Fig. 2.3 The C_5 pathway of ALA formation in Arabidopsis chloroplasts

Chapter 3. Materials and Methods

3.1. Plant materials

Narcissus bulbs (*Narcissus tazetta L.*) imported from China were purchased from a local nursery. Fresh bulbs were either used immediately for extraction of slimy substances or freeze-dried and stored for later used.

Seeds used in biochemical and physiological effects of NCS were Chinese cabbage (*Brassica parachinese*), rice (*Oryza sativa L.*), barley *(Hordeum vulgare*) and radish (*Raphanus sativus V.*) purchased locally in Hong Kong, and wheat (*Triticum aestivum cv.* Lonchun No.10) purchased from Gansu Agricultural Academy.

3.2. Isolation and purification of inhibitory substance from *Narcissus* bulbs

I. Isolation of inhibitory substance from fresh *Narcissus* bulbs

After the removal of the outermost dry scales and roots, fresh bulbs were chopped into small pieces and placed in a glass basin and soaked with water. Slimy substances were secreted from the cut surfaces of bulbs within several hours and remained in the water for at least five days. The slimy secretion was collected every 30 hours for 4 times. The slimy

secretion was pooled and dried in vacuum at -40°C. The lyophilized slimy secretion was stored in a desiccator for further isolation, purification and identification.

II. Partial purification of the inhibitory substance with different organic solvents

Freeze-dried bulbs or lyophilized slimy secretion (LSS) packed in a large filter paper tumbler were put in a Soxhlet extractor, then were either extracted with the following organic solvents: cyclohexane, ethyl acetate, ethanol and water; or were directly extracted with n-butanol.

In the latter part of this investigation, freeze-dried bulbs or LSS were also extracted with 75% ethanol. The filtered extracts were concentrated at 40°C in vacuum. The residue was then extracted with an equal volume of petroleum ether for five times. The same procedure was followed by using ethyl acetate and n-butanol.

The presence of an inhibitor of each fraction was assayed by the *Brassica* seed germination test.

III. Purification and Identification

A. Thin layer chromatography (TLC)

An aliquot of the extract (200µl) with n-butanol was spotted on TLC plate (either Art. 5745, Kieselgel 60 with 2mm thickness or Art. 5714, Kieselgel 60 F_{254}) and developed with AcOEt : MeOH (100:5;v/v).

B. Column chromatography

Silica gel (9385 9025, Kieselgel 60) column (5.0x30cm^2) was used for further purification. This process used stepwise elution with AcOEt and with increasing concentration of , (AcOEt:MeOH = 100:0, 100:1, 100:2, 100:4, 100:5, 100:10, 100:20, 100:50, 100:100 and 0:100). The presence of inhibitory substance in *Narcissus* bulbs in each fraction during extracting was determined by *Brassica* seed germination test. The active fraction was chromatagraphed on a second column of the same silica gel (2.5x45cm^2) and eluted with mixtures of dichloromethane and methanol (dichloromethane:methanol = 100:0, 100:1, 100:2, 100:4 and 100:5, v/v).

C. Spectrometric Analyses

Infrared (IR) spectra was obtained in KBr with a Nicolet FTIR 205 spectrometer. Ultraviolet (UV) spectra was determined in methanol with a DU-7 spectrophotometer (Beckman). ^{1}HNMR spectra was recorded at Brucker - 250. MS spectral was obtained on the VG - 7070F mass spectrometer.

Crystal structure of the inhibitory substance was determination by X-ray analysis. Intensities were collected in the variable ω-scan technique (Sparks, 1976) on a Siemens R3m/V diffractometer using MoKα radiation(λ=0.710773A) at 293 K. The raw data were processed with a learnt-profile procedure (Kopfmann and Huber, 1968). Direct methods yielded the positions of all non-hydrogen atoms. The hydrogen atoms on the carbon atoms were generated geometrically (C-H fixed at 0.96A) and allowed to ride on their respective parent atoms, and the other hydrogen atoms were located from subsequent difference Fourier syntheses. All hydrogen atoms were assigned appropriate isotropic temperature factor and included in the structure-factor calculations.

IV. Bioassays

Various volumes of solution from the different fractions were put onto a filter paper in a 3.3cm Petri dish, and then dried with hot air. One-ml distilled water was then added in each Petri dish. Twenty seeds of *Brassica* were put in each dish and kept in the dark for two or three days. The seeds were recorded as germinated when 1mm radical was visible. The number of germinating seeds and the length of radicle or hypocotyl were recorded. Each treatment was triplicated.

3.3 Effect of narciclasine (NCS) on seed germination and seedling

growth

I. Germination experiments

Fifty ml of different concentrations of NCS was put onto a Whatman No.1 filter paper in a 3.3cm Petri dish and then dried with hot air. One-ml distilled water or different concentrations of ABA solutions was added to each Petri dish. The final concentrations of NCS were 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M. Twenty seeds (Chinese cabbage or radish) or ten seeds (rice) were used for the germinability test. The seeds were recorded as germinated when 1mm radicle was visible. The number of germinating seeds and the length of radicles and hypocotyls or coleoptiles were recorded and six replicates were used in each treatment.

II. Seedling growth

Lengths of radicles and hypocotyls of radish seedlings were measured after 36, 48 and 60 hr imbibition in the dark.

The elongation of radicle and hypocotyl of *Brassica parachinese* seedlings was measured after 48, 60 and 72 hr imbibition in the dark.

Lengths of radicles and coleoptiles of rice seedlings were measured after 72 hr imbibition in dark.

3.4. Interaction of NCS and phytohormones

I. Interaction with abscisic acid (ABA)

The seed germination and seedling growth of *Brassica* was used to demonstrate the interaction of NCS and ABA.

A. Seed germination

Twenty *Brassica* seeds were allowed to germinate in a 3.3cm Petri dish containing various concentrations of NCS, ABA or both of them up to 72 hr in dark. The seeds were recorded as germinated when 1mm radicle was visible. The number of germinating seeds was recorded at different times during 72 hr germination in dark and six replicates were used in each treatment.

B. Seedling growth

Lengths of radicles and hypocotyls of *Brassica* seedlings were measured after 48 hr, 60 hr and 72 hr germinating in dark. Six replicates were used in each treatment.

II. Interaction with auxin

The wheat coleoptile section was used to demonstrate the interaction of NCS and auxin. Wheat seeds after 20 hr imbibition in the dark at 22±2°C were incubated for 72 hr at 28°C in darkness. Ten 5mm coleoptile sections of wheat seedlings were incubated in 1ml 1% sucrose solution (10mM phosphate-citrate, pH5.4) containing various concentrations of indole-3-acetate (IAA) and NCS in a 3.3cm Petri dish with Whatman No.1 filter paper for 20 hr at 28°C. The length of coleoptile sections was measured and 6 replicates were used in each treatment.

III. Interaction with Gibberellin

The barley endosperm bioassay was used to demonstrate the interaction of NCS and gibberellin. The release of reducing sugars from the endosperms of barley half-seeds was assayed according to the method described by Sakoda (1991). Barley seeds after surface sterilization with 15% bleach for 20 min, were rinsed ten times in distilled water. The seeds were cut transversely 3mm from the distal end with a razor and the embryo-containing fragments were discarded. Ten embryo-free endosperm fragments were placed in a 10ml vial containing 1.0ml of the test solution with 0.5mg Streptomycin. Different concentrations of GA_3, NCS or both were included as required. The stoppered vials were placed in a shaking incubator for 48 hr at 30°C in darkness. The sample solutions were diluted to 10ml with distilled water and centrifuged for 10 min at 3000rpm (ECCO-PRAXA-Z). One-ml supernatant was added to Somogyi's alkaline copper reagent in a test tube and placed in a

boiling water bath for 10 min. After cooling, 1ml of Arsenomolybdate reagent was added, mixed thoroughly, and then diluted to 10ml. Absorbance at 560nm was recorded. α-amylase activity was expressed as μg glucose/seed.

IV. Interaction with cytokinin

The radish cotyledons expansion assay was used to demonstrate the interaction of NCS and cytokinin. Seeds of radish were sown on two layers of Whatman No.1 filter paper moistened with distilled water in a Petri dish, and allowed to germinate in darkness for 48 hr at 22±2°C. Cotyledons of uniform size were excised from seedlings. Ten excised cotyledons were transferred to a 3.3cm Petri dish. Each Petri dish contained a layer of Whatman No.1 filter paper moistened with 1ml distilled water containing various concentrations of BA, NCS or both BA and NCS. After 48hr incubation at 28°C under continuous light (50 uEm^{-2} s^{-1} PAR) or darkness, the cotyledons were blotted with filter paper and the fresh weight or chlorophyll contents were measured.

Fresh weights of excised radish cotyledons exposed to the light or the dark for 48 hr were measured.

After 48 hr in the light, the excised cotyledons were weighed, then frozen with liquid nitrogen and stored at -20°C. Frozen cotyledons were ground with 80% acetone in a mortar. Each extract was then centrifuged for 10 min at 3000rpm (ECCO-PRAXA-Z). The final volume of supernatant was made into 5ml with 80% acetone. Chlorophyll content was

measured spectrophotometrically at 645nm and 663nm, and calculated according to the method of Arnon (1949). Results were expressed in mg chlorophyll per cotyledon.

For carotenoid content determination, excised cotyledons from radish seedlings germinating in the dark for 48 hr at 22±2°C were incubated in the dark for 48 hr at 28°C. The cotyledons were blotted with filter paper, weighed, then frozen at -20°C. The cotyledons were ground and extracted with 100% acetone in a mortar. Each extract was then centrifuged for 10min at 3000rpm (ECCO-PRAXA-Z). The final volume of supernatant was made into 5ml with 100% acetone. Carotenoid were assayed spectrophotometrically at 450nm. The calculation of carotenoid content was according to the method described by Longo (1979) and expressed in μg/cotyledon.

3.5. Interaction of NCS and phytohormones to growth and greening of excised radish cotyledons exposing to light

I. Growth of excised radish cotyledons exposing to light

The growth of cotyledons was estimated by the increase of fresh weight (average of the fresh weight of ten cotyledons was about 70mg). Ten randomly selected cotyledons were blotted gently, weighed as a group and placed on Whatman No. 1 filter paper with the adaxial side down covering the bottom of a 3.3cm Petri dish containing different concentrations of NCS and various phytohormones or both of them as required. The Petri

dishes were placed under light for 48 hr at 28°C. After incubation, cotyledons were blotted with filter paper and the fresh weights were measured. The difference of fresh weights was expressed as the growth of excised radish cotyledons.

II. Chlorophyll content determination

The cotyledons immediately after excision were weighed and then were frozen with liquid nitrogen and stored at -20°C. The frozen samples were homogenized with 5ml 80% acetone in a mortar. The extracts were centrifuged for 10 min at 3000rpm (ECCO-PRAXA-Z). The absorbance of supernatant was measured at 645nm and 663nm. The chlorophyll contents were calculated according to the method described by Arnon (1949).

III. Effects of a pretreatment with BA or NCS on the growth and greening of excised radish cotyledons

Pretreatments with BA or NCS were performed immediately after excision of the radish cotyledons. The excised cotyledons immediately after excision were incubated on a filter paper containing 1ml 10^{-5}M BA or 10^{-6}M and 10^{-5}M NCS solutions for different times (5 min to 4 hr) in a petri dish. At the end of the incubation period, the cotyledons were dried with filter paper, rinsed in distilled water for three times, and then they were incubated in water and NCS (10^{-6}M and 10^{-5}M) solutions, or in water and BA (10^{-5}M) solution, respectively, for 48 hr in the light at 28°C.

3.6. Effect of NCS on the growth and greening of excised radish cotyledons and etiolated wheat leaves

I. Effect of NCS on the growth and greening of excised radish cotyledons

Radish seeds were sown on two layers of Whatman No.1 filter paper moistened with distilled water in Petri dishes, and allowed to germinate in the dark for 48 hr at 22±2°C. Ten randomly selected cotyledons were excised from seedlings. They were blotted, weighed and placed, with adaxial side down, on Whatman No.1 filter paper covering the bottom of a 3.3cm Petri dish containing various concentrations of NCS as required. They were incubated either in light or dark for 12, 24, 36, 48 and 60 hr at 28°C. At different time intervals, cotyledons were blotted with filter paper and the fresh weights were recorded. The difference of fresh weights was expressed as the growth of excised cotyledons. Those excised cotyledons cannot be assayed immediately for chlorophyll contents were frozen with liquid nitrogen and stored at -20°C.

II. Effect of NCS on the greening of etiolated wheat leaves

Wheat (*Triticum aestivum, cv.* Longchun No.10) seeds after surface sterilization with 15% bleach for 20min, were rinsed ten times with distilled water and allowed to

imbibe for 20 hr in the dark. Seeds showing emerged radicle were planted in vermiculite and incubated at 25°C in the dark. Distilled water was added once every 2 days. wheat seedlings (7-day-old) were selected for chlorophyll study. The primary leaves of 7-day-old etiolated wheat seedlings were excised under a green safe-light and floated in distilled water for 2 to 4 hr to ensure the turgidity throughout the experiment according to the method described by Nadler and Granick (1970). Eight 2.5cm leaf-sections, excised from each leaf with the top 0.5cm removed, were placed in a 3.3cm Petri dish with Whatman No.1 filter paper containing various concentrations of NCS as required. After exposure to light for 12, 24, 36 and 48 hr, the chlorophyll contents of the leaf sections was determined according to the method described by Arnon (1949). The chlorophyll content was expressed as μg/leaf section.

3.7. Effect of NCS on chlorophyll synthesis and δ-aminolevulinic acid (ALA) accumulation of etiolated wheat leaves in the presence of levulinic acid (LA) under light

Leaf sections of 7-day-old etiolated wheat seedlings were used to study chlorophyll and ALA production exposed to light for 12, 24, 36 and 48 hr. Eight sections of etiolated wheat leaf were placed in a 3.3cm Petri dish containing distilled water or different concentrations of NCS and LA solution (pH adjusted to pH6.8 with NaOH) as required for 3 hr before the onset of illumination without vacuum infiltration as described by Dei (1985).

All manipulations were performed under dim green light. After the dark preincubation, etiolated wheat leaf sections were continuously irradiated under light for 2, 4, 8, 16 and 24 hr. Then leaf sections were blotted and weighed. After that, they were frozen with liquid nitrogen and stored at -20°C until being used to measure chlorophyll and ALA contents. Chlorophyll extraction and determination were carried out as described previously.

For the determination of ALA, wheat leaf sections were homogenized with 5ml of 4% trichloroacetic acid (w/v). The homogenate was centrifuged at 3000rpm (ECCO-PRAXA-Z) for 10 min. One-ml of the supernatant was added to a test tube containing 1ml 4% trichloroacetic acid (w/v), 0.94ml of 1M sodium acetate and 0.06ml of acetylacetone. The mixture was brought to boiling for 15 min. After cooling, equal aliquots of the sample and modified Ehrlich's reagent (1g of DMAB in 10ml of 70% perchloric acid and 40ml of glacial acetic acid) were mixed as described by Mauzerall and Granick (1956). After 15 minutes, the ALA content in the reaction mixture was determined by recording absorbance at 554nm.

3.8. Enzymes studies in the excised radish cotyledons

Crude extracts for determination of total enzyme activities were prepared according to the methods described by Servettaz et al. (1976). Thirty excised cotyledons from radish seedlings after being exposed to the light for different time intervals up to 48 hr were ground in a mortar with 6ml of medium (50mM K-phosphate, pH7.4; 2mM Na-EDTA;

10mM mercaptoethanol). The homogenate was squeezed through four layers of cheesecloth and centrifuged at 28,000g (Beckman J2-M1) for 20 min. The supernatant was carefully removed without disturbing the fat layer that floated on the top of the centrifuge tube and used for enzyme activities assay.

I. Assay of isocitrate lyase activity

Isocitrate lyase activity was assayed spectrophotometrically by the method of Dixon and Kornberg (1959). The principle of this assay depends on measurements of the rate of increase of absorbance at 324nm. The assay mixture (3ml) contained 200μmoles of potassium phosphate (pH6.85), 15μmoles of $MgCl_2$, 10μmoles of phenylhydrazine-HCl, 6μmoles of cysteine HCl, and 1.0ml supernatant. The reaction is started by the addition of 5μmoles of potassium isocitrate and absorbance at 324nm was followed for 5 min. The rate is proportional to the isocitrate lyase concentration. Isocitrate lyase activity was calculated following the method described by Dixon and Kornberg (1959). The activity of isocitrate lyase was expressed as μmoles glyoxylate formed/cotyledon/min.

II. Assay of hydroxypyruvate reductase activity

The activity of hydroxypyruvate reductase was measured by the decrease of absorbance at 340nm in the presence of hydroxypyruvate. The assay mixture contained 200μl of 0.02M phosphate buffer (pH6.2), 50μl $4X10^{-4}$M NADH, 30μl 0.5% Triton X-100 and 520μl of water and 100μl supernatant. The reaction was initiated with 100μl 0.01M

hydroxypyruvate and absorbance at 340nm was followed for 5min as the reaction rate of enzyme catalyzed. The activity of hydroxypyruvate reductase was expressed as μmoles NADH oxidized/cotyledon/min (Tolbert, 1970).

3.9. Ultrastructural studies

Excised radish cotyledons after germinating for 48 hr were transferred to a 3.3cm Petri dish. Each Petri dish contained a filter paper moistened with 1ml distilled water and placed either in the light or the dark for different times as required. Then they were used for electronic microscopic examinations.

For electron microscopy, small pieces (about 1x1x3 mm^3) of excised radish cotyledons after different treatments were cut from the cotyledons halfway between the central vein and the edge. They were fixed and embedded according to the method as described by Longo et al. (1979) with slight modification. The protocol was followed as shown in Table 3.1. The pieces were fixed in 3% glutaraldehyde in 0.05M phosphate buffer (pH7.0) for 12hr in a vacuum. After three times washings in 0.05M a phosphate buffer (pH7.0) the material was postfixed with 2% OsO4 for 2hr and three 10min washes in K-phosphate buffer. The materials were dehydrated in a graded series of ethanol infiltrated in a vacuum, and embedded in a low-viscosity resin (Spurr, 1969). Sections were cut with a Reichart ultratome, stained with uranyl acetate and lead citrate, and were viewed with a Zeiss EM 9S-2 electron microscope.

Table 3.1 Tissue preparation procedure for TEM

PROCEDURE	REAGENT	TIME	TEMPERATURE
Fixation in vacuum	3% glutaraldehyde in K-phosphate buffer pH7.0	3hrs	4°C
Buffer wash	K-phosphate buffer	5min	R.T
	K-phosphate buffer	5min	R.T
Post fixation	2% Osminum tetroxide in K-phosphate buffer	2hrs	R.T
Buffer wash	K-phosphate buffer	5min	R.T
	K-phosphate buffer	5min	R.T
Dehydration in vacuum	50% Ethanol	10min	R.T
	70% Ethanol	10min	R.T
	85% Ethanol	10min	R.T
	95% Ethanol	15min	R.T
	95% Ethanol	15min	R.T
	100% Ethanol	15min	R.T
	100% Ethanol	15min	R.T
Infiltration in vacuum	100% Ethanol:Spurr (2:1)	4hrs	R.T
	100% Ethanol:Spurr (1:1)	4hrs	R.T
	100% Ethanol:Spurr (1:2)	12hrs	R.T
	Pure spurr	3hrs	R.T
	Pure spurr	3hrs	R.T
	Pure spurr	3hrs	R.T
Embedding	Pure spurr	12hrs	68°C

Chapter 4. Results

4.1. Chemical studies of NCS

I. Isolation and partial purification of inhibitory substance from *Narcissus* bulbs

A. Effect of lyophilized slimy secretion (LSS) on the germination, the growth of radicle and hypocotyl of seedlings of *Brassica*

LSS prepared according to procedure described in section 3.2, was re-dissolved in distilled water. Fig.4.1 showed the effect of different concentrations of LSS on the germination and elongation of radicle of *Brassica* seeds. The inhibitory effect of LSS on seed germination and elongation of radicle of *Brassica* increased with increasing concentration of LSS. At 2.4mg/ml of LSS, both germination and radicle elongation were totally inhibited.

B. Effect of different solvent extracts on the germination of *Brassica* seeds and the elongation of radicle and hypocotyl of *Brassica* seedlings

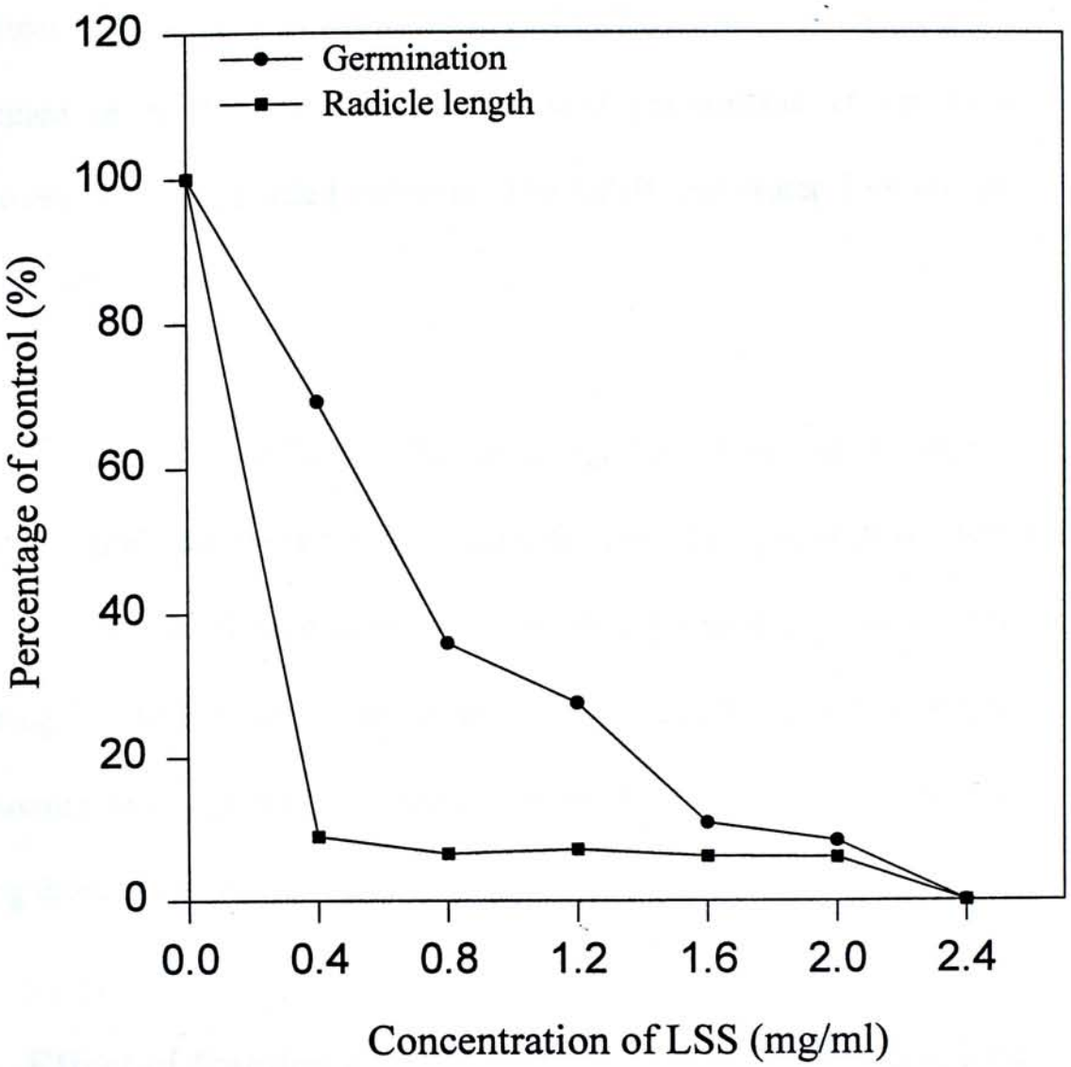


Fig. 4.1 Effect of LSS on germination of seed and radicle elongation of seedlings of *Brassica*.

Dry bulbs of *Narcissus* were used to isolate the inhibitory substance. Table 4.1 showed the effects of various concentrations of different solvent extracts (cyclohexane, ethyl acetate, ethanol and water) on the germination of *Brassica* seeds. Results revealed that the ethyl acetate fraction caused the most potent inhibition on seed germination as compared with water control. Compared to the water control, the ethyl acetate fraction resulted in an 82.6% inhibition on seed germination at the extract concentration equivalent to 60mg dried bulbs/ml. The EtOH and water fractions also showed strong inhibitory effects.

As shown in Table 4.2, the elongation of radicle of *Brassica* seedlings was strongly inhibited by the ethyl acetate fraction. Compared to the water control, 96.4% inhibition on the elongation of radicle of *Brassica* seedlings was observed in this fraction during incubation for 72 hr in the dark at 22±2°C. The elongation of hypocotyl of *Brassica* seedlings was completely inhibited by the ethyl acetate extract equivalent to 6mg dried bulbs/ml concentration (Table 4.3).

C. Effect of fraction isolated with n-butanol from dried bulbs or LSS on the germination of *Brassica* seeds and radicle growth

Table 4.4 showed that the inhibitory substance can also be directly extracted with n-butanol. There was an approximately 94.1% inhibition on germination of *Brassica*

Table 4.1 Effect of different extracts from dry bulbs of *Narcissus* on *Brassica* seed germination

Concentration[1] (mg/ml)	Percentage[2] (%) of seed germination			
	cyclohexane	AcOEt	95% EtOH	H_2O
0[3]	89.0	89.0	89.0	89.0
6	74.0	74.6	77.5	90.0
12	75.0	75.0	69.0	91.2
24	75.4	61.5	70.5	86.5
48	74.5	34.0	67.0	75.0
60	73.0	15.5	53.0	50.3

[1] Equivalent to mg dry bulb /ml
[2] No. of germinated seeds / total no. of seeds
[3] Water control

The fraction was obtained by extracting either with cyclohexane, AcOEt, EtOH or water, respectively, in Soxhlet Extractor from dried bulbs of *Narcissus*. *Brassica* seeds were allowed to germinate in different concentrations of various extracts from dried bulbs at 22±2°C in dark. The number of germinating seeds of *Brassica* was recorded when 1mm radicle was visible after incubation for 72 hr.

Table 4.2 Effect of different extracts from dry bulbs of *Narcissus* on *Brassica* radicle elongation

concentration[1] (mg/ml)	Length of radicle							
	Cyclohexane		AcOEt		95% EtOH		H_2O	
	mm	%[2]	mm	%	mm	%	mm	%
0	27.5	100.0	27.5	100.0	27.5	100.0	27.5	100.0
6	25.7	93.5	3.7	13.5	4.7	27.1	22.5	81.8
12	24.8	90.2	2.0	7.3	2.0	12.8	2.0	12.8
24	24.5	89.1	1.4	5.1	2.2	8.0	1.7	6.2
48	22.1	80.4	1.1	4.0	1.3	4.7	1.4	5.1
60	21.2	77.1	1.0	3.6	1.0	3.6	1.2	4.4

[1] Equivalent to mg of dry bulb per ml

[2] % of water control

Table 4.3 Effect of different extracts from dry bulbs of *Narcissus* on *Brassica* hypocotyl elongation

Concentration[1] (mg/ml)	Length of hypocotyl							
	Cyclohexane		AcOEt		95% EtOH		H_2O	
	mm	%[2]	mm	%	mm	%	mm	%
0	11.0	100.0	11.0	100.0	11.0	100.0	11.0	100.0
6	8.5	77.2	0.0	0.0	4.5	40.9	9.5	86.4
12	8.5	77.2	0.0	0.0	2.2	20.0	0.0	0.0
24	7.2	65.5	0.0	0.0	0.0	0.0	0.0	0.0
48	6.4	58.2	0.0	0.0	0.0	0.0	0.0	0.0
60	6.4	58.2	0.0	0.0	0.0	0.0	0.0	0.0

[1] Equivalent to mg of dry bulb per ml

[2] % of water control

Table 4.4 Effect of n-butanol extract from dry bulbs of *Narcissus* on the germination and radicle elongation of *Brassica* seeds

Concentration[1] (mg/ml)	Percentage of germination		Length of radicle	
	No.	%[2]	mm	%[3]
0	17.0	85.0	13.6	100.0
25	2.4	12.0	1.8	12.2
50	1.0	5.0	1.1	8.1
75	0.0	0.0	0.0	0.0

[1]Equivalent to mg of dry bulb per ml

[2] No. of germinated seeds / total no. of seeds

[3] % of water control

seeds at concentration equivalent to 50mg dried bulbs/ml after incubation for 48 hr in dark at 22±2°C. The inhibition of this fraction on radicle growth of *Brassica* seedlings was about 91.9% at 50mg dried bulbs/ml.

For LSS (Table 4.5), the fraction extracted with n-butanol (equivalent to 2.4mg/ml) caused an 81.9% inhibition on germination of *Brassica* seeds during incubation for 48 hr in the dark at 22±2°C. An approximately 92.1% inhibition was observed for the radicle growth of *Brassica* seedlings.

D. Purification of inhibitory substance from *Narcissus* bulbs by chromatography

The results (Fig.4.2) obtained with prepared TLC (thin layer chromatography) developed in AcOEt:MeOH (100:5) indicated that the germination of *Brassica* seeds was markedly inhibited by fractions extracting by n-butanol from LSS with 0.00-0.18 and 0.18-0.43 of R_f values at 1x and 2x concentrations equivalent to 2.4mg/ml and 4.8mg/ml of LSS during incubation for 48 hr in the dark at 22±2°C. The inhibitions were up to about 53.8% and 69.2% at 2x concentrations. The radicle elongation was also obviously inhibited by the fractions of R_f 0.00 - 0.18 and R_f 0.18 - 0.43 (Fig. 4.3). There were about 87.7% and 88.8% inhibition to radicle elongation of *Brassica* seedlings at 2x

Table 4.5 Effect of n-butanol extract from LSS of *Narcissus* on the germination and radicle elongation of *Brassica* seeds

Concentration[1] (mg/ml)	Percentage of germination		Length of radicle	
	No.	%[2]	mm	%[3]
0	16.6	83.0	12.7	100.0
0.3	15.1	75.5	13.0	102.4
0.6	13.0	65.0	6.3	49.6
1.2	5.3	26.5	1.4	11.0
2.4	3.0	15.0	1.0	7.9
3.0	0.0	0.0	0.0	0.0

[1]Equivalent to mg LSS per ml

[2] No. of germinated seeds / total no. of seeds

[3] % of water control

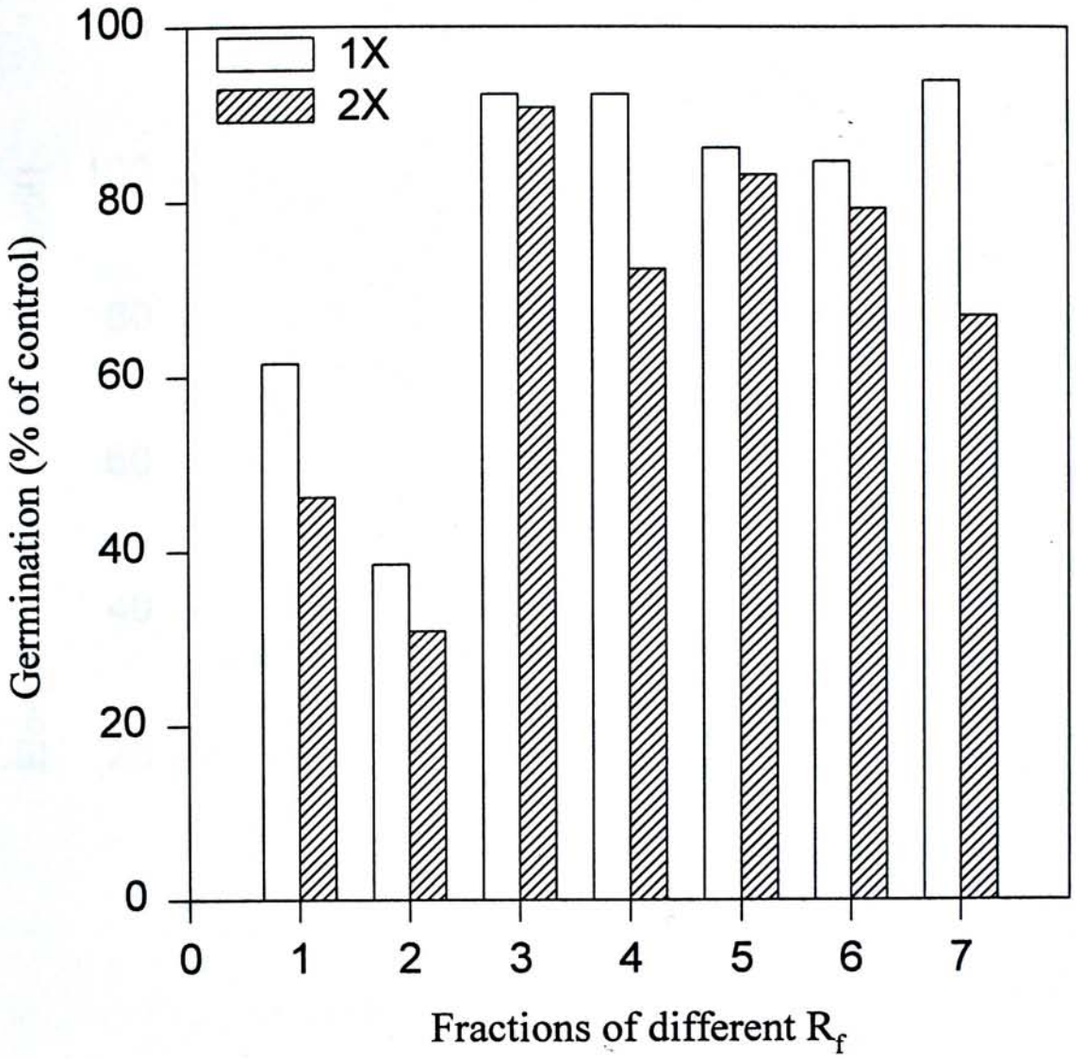


Fig. 4.2 Effect of different fractions of extract on the germination of *Brassica* seeds. The crude material was extracted directly by n-butanol was further separated on preparative TLC with AcOE : MeOH (100 : 5; v/v).

1: R_f 0.00-0.18
2: R_f 0.18-0.43
3: R_f 0.43-0.50
4: R_f 0.50-0.60
5: R_f 0.60-0.76
6: R_f 0.76-0.92
7: R_f 0.92-1.00

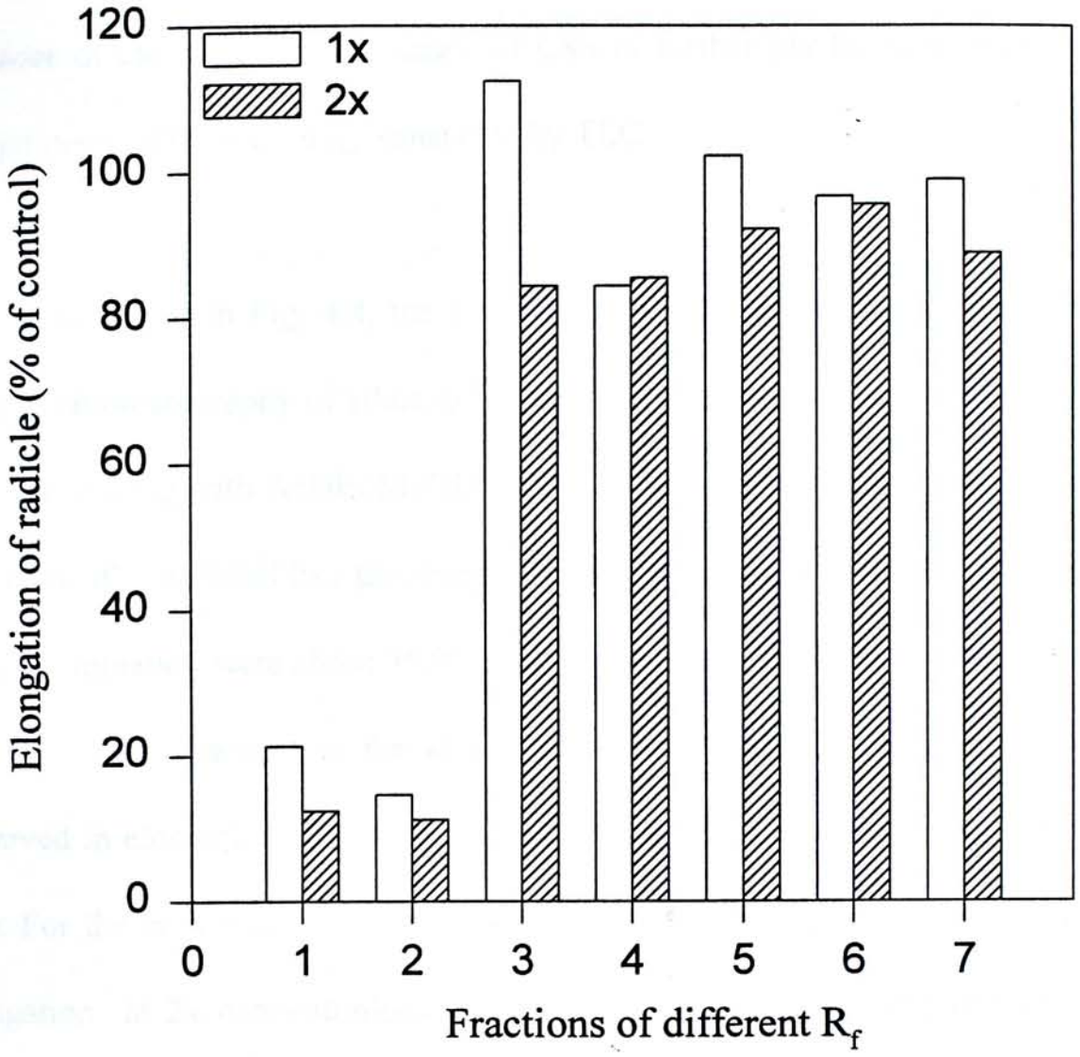


Fig. 4.3 Effect of different fractions of extract on the radicle elongation of *Brassica* seedlings. The crude material was extracted by n-butanol and was further separated as described in Fig. 4.2.

1: R_f 0.00-0.18
2: R_f 0.18-0.43
3: R_f 0.43-0.50
4: R_f 0.50-0.60
5: R_f 0.60-0.76
6: R_f 0.76-0.92
7: R_f 0.92-1.00

concentrations equivalent to 2.4mg LSS /ml during cultivation for 48 hr in dark at 22±2°C. Moreover, under 366nm light, R_f 0.18-0.43 fraction exhibited a strong yellow green fluorescence stimulated by 366nm of light. So did in dried bulbs. Therefore, the presence of the inhibitory substance of LSS in further purification was detected using this property of the inhibitory substance by TLC.

As shown in Fig. 4.4, the inhibitions of various eluting fractions extracted with column chromatography of silica gel to seed germination of *Brassica* were mainly in the fractions eluting with AcOEt:MeOH (100:1, 100:2). The germination of *Brassica* seeds was markedly inhibited like the result obtaining with preparative TLC, their inhibitions to seeds germination were about 55.9% and 35.3% at 2x concentrations equivalent to 2.4mg LSS /ml during incubation for 48 hr under dark at 22±2°C. Similar phenomena were observed in elongation of radicle or hypocotyl of *Brassica* seedlings. (Fig. 4.5 and Fig. 4.6). For the radicle growth, there were around 88.7% and 92.5% inhibitions to radicle elongation at 2x concentration in AcOEt:MeOH was 100:1 and 100:2 (v/v) fractions (Fig. 4.5). For the hypocotyl growth, however, 100% inhibition was exhibited in these two fractions (Fig. 4.6). On the TLC detection with developing in AcOEt:MeOH (100:5), all the active fractions exhibited a yellow green fluorescence point under 366nm. After that, these active fractions were chromatographed on a column of the same silica gel with dichloromethane:methanol solvent system in increasing concentration of methanol. The fraction which was eluted by 5-6% methanol only exhibited the yellow green fluorescence point on the TLC detection. The yellowish crystals were obtained after

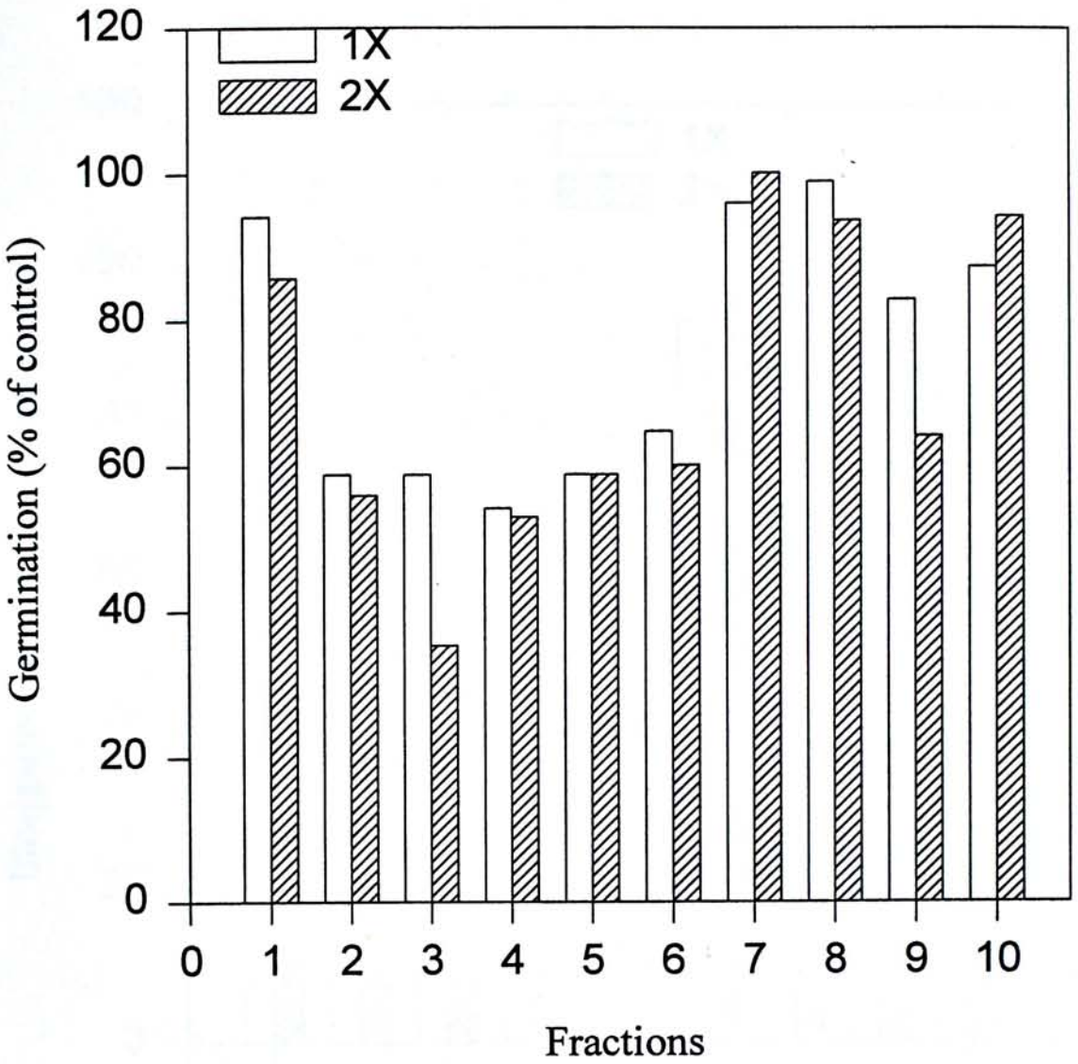


Fig. 4.4 Effect of different fractions on the germination of *Brassica* seeds. *Brassica* seeds were allowed to germinate in different fractions at 1x or 2x concentration equivalent to 2.4mg/ml or 4.8mg/ml of LSS. The AcOEt fraction of LSS isolated by Soxhlet Extractor was separated by silica gel column chromatography using AcOEt : MeOH system in increasing concentration of methanol.

AcOEt : MeOH;	1:	100:0	6:	100:10
	2:	100:1	7:	100:20
	3:	100:2	8:	100:50
	4:	100:4	9:	100:100
	5:	100:5	10:	0:100

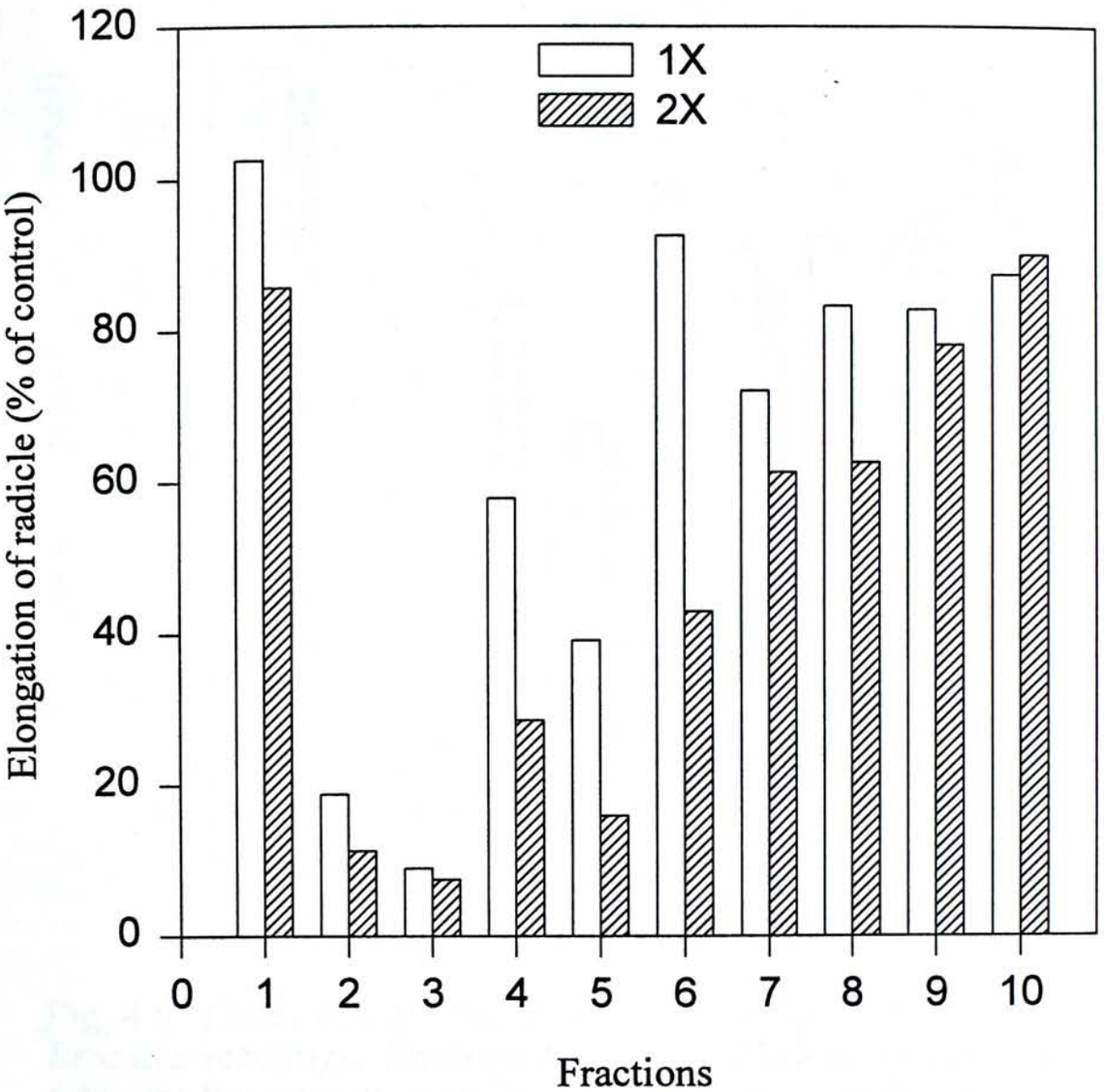


Fig. 4.5 Effect of different fractions on the radicle elongation of *Brassica* seedlings. *Brassica* seeds were allowed to germinate in different fractions at 1x or 2x concentration equivalent to 2.4mg/ml or 4.8mg/ml of LSS. The AcOEt fraction of LSS was separated as described in Fig. 4.4.

AcOEt : MeOH;

1:	100:0	6:	100:10
2:	100:1	7:	100:20
3:	100:2	8:	100:50
4:	100:4	9:	100:100
5:	100:5	10:	0:100

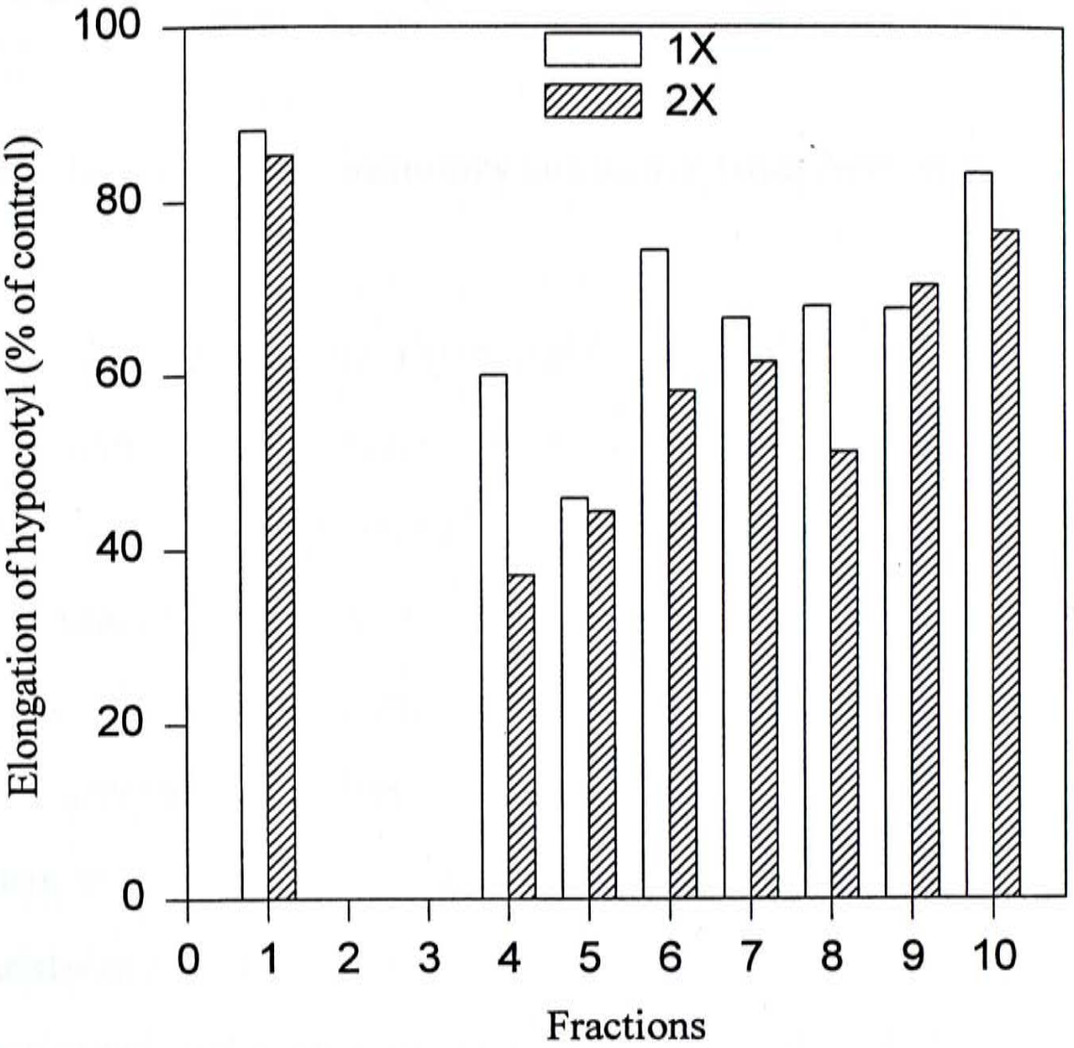


Fig. 4.6 Effect of different fractions on the hypocotyl elongation of *Brassica* seedlings. *Brassica* seeds were allowed to germinate in different fractions at 1x or 2x concentration equivalent to 2.4mg/ml or 4.8mg/ml of LSS. The AcOEt fraction of LSS was separated as described in Fig. 4.4.

AcOEt : MeOH;

1:	100:0	6:	100:10
2:	100:1	7:	100:20
3:	100:2	8:	100:50
4:	100:4	9:	100:100
5:	100:5	10:	0:100

allowing to evaporate the eluted solvent standing at room temperature for three to four weeks (Fig. 4.7).

II. dentification of the inhibitory substance from *Narcissus* bulbs

IR (cm^{-1}) spectrum (Fig. 4.8) showed the peaks at 3400, 1660, 1460, 1380, 1080, 1030, 920 (methylenedioxy group). The UV spectrum (Fig. 4.9) had a peak at 252.3nm. The MS spectrum (Fig. 4.10) showed the peak of molecular ion MS (EI) m/e 307 (M^+, 25), and malor peaks of 289 (8), 271 (15), 247 (100), and 218 (18). The ^{1}HNMR spectral assignment for this substance was given in Fig. 4.11. These data were similar to that obtained by Piozzi et al. in 1968, and it was named as narciclasine by Ceriotti in 1967. Therefore, the inhibitory substance from LSS or dried bulbs of *Narcissus* was identified as narciclasine and its structure is as Fig. 4.12A (Piozzi et al., 1968). However, the structure of narciclasine was corrected on the basis of a reinterpretation of the ^{1}HNMR spectrum of the O- methyl-triacetyl derivative by Mondon and Krohn in 1970 (Fig. 4.12B). As Fig. 4.13 shown, the result of X-ray analysis to single crystal structure was also indicated the structure of narciclasine is similar to that described by Mondon and Krohn in 1970 (Fig. 4.12B).

Fig. 4.7 Crystals of inhibitory substance isolated from *Narcissus* bulbs

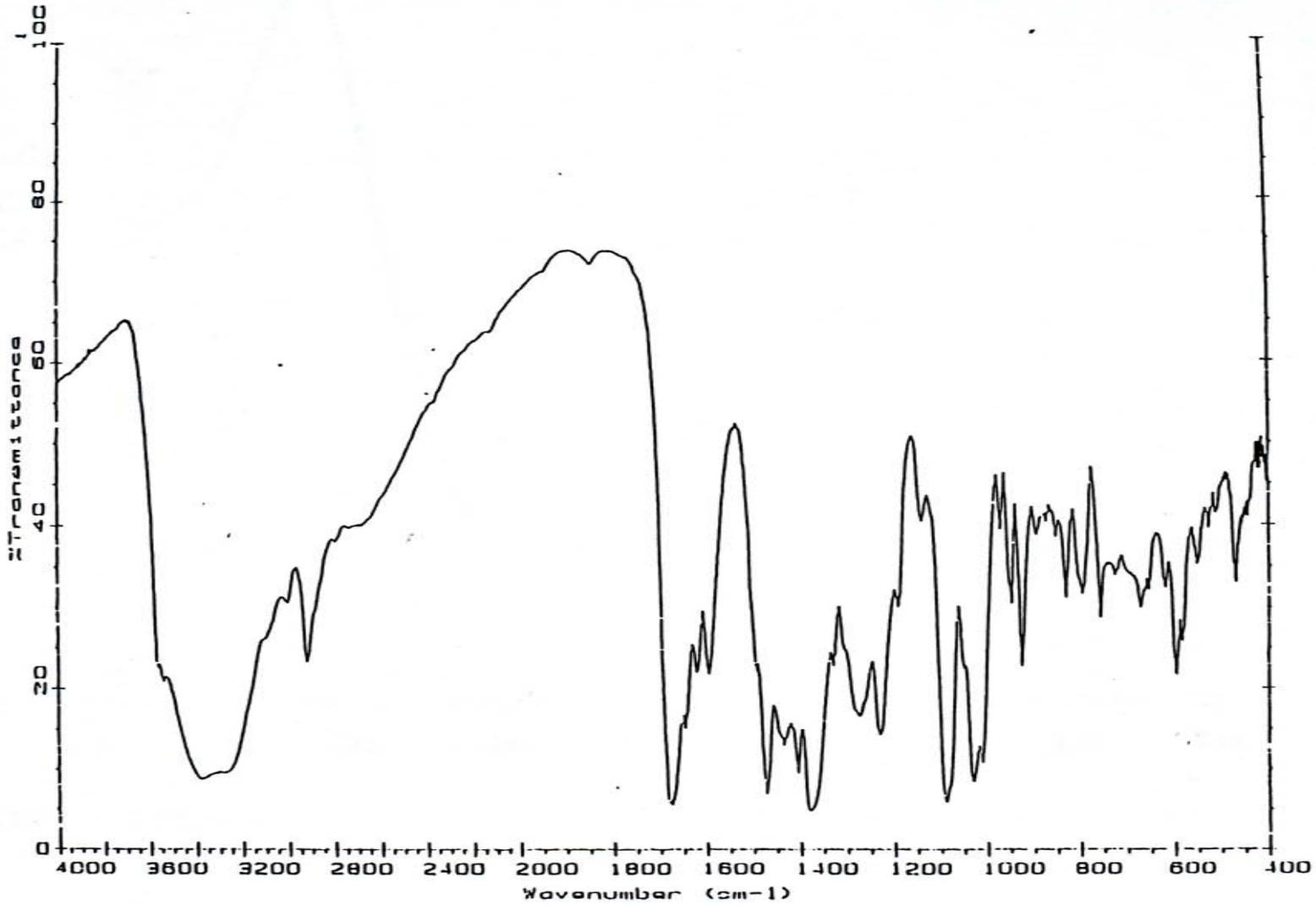


Fig. 4.8 IR spectrum of the inhibitory substance isolated from *Narcissus* bulbs

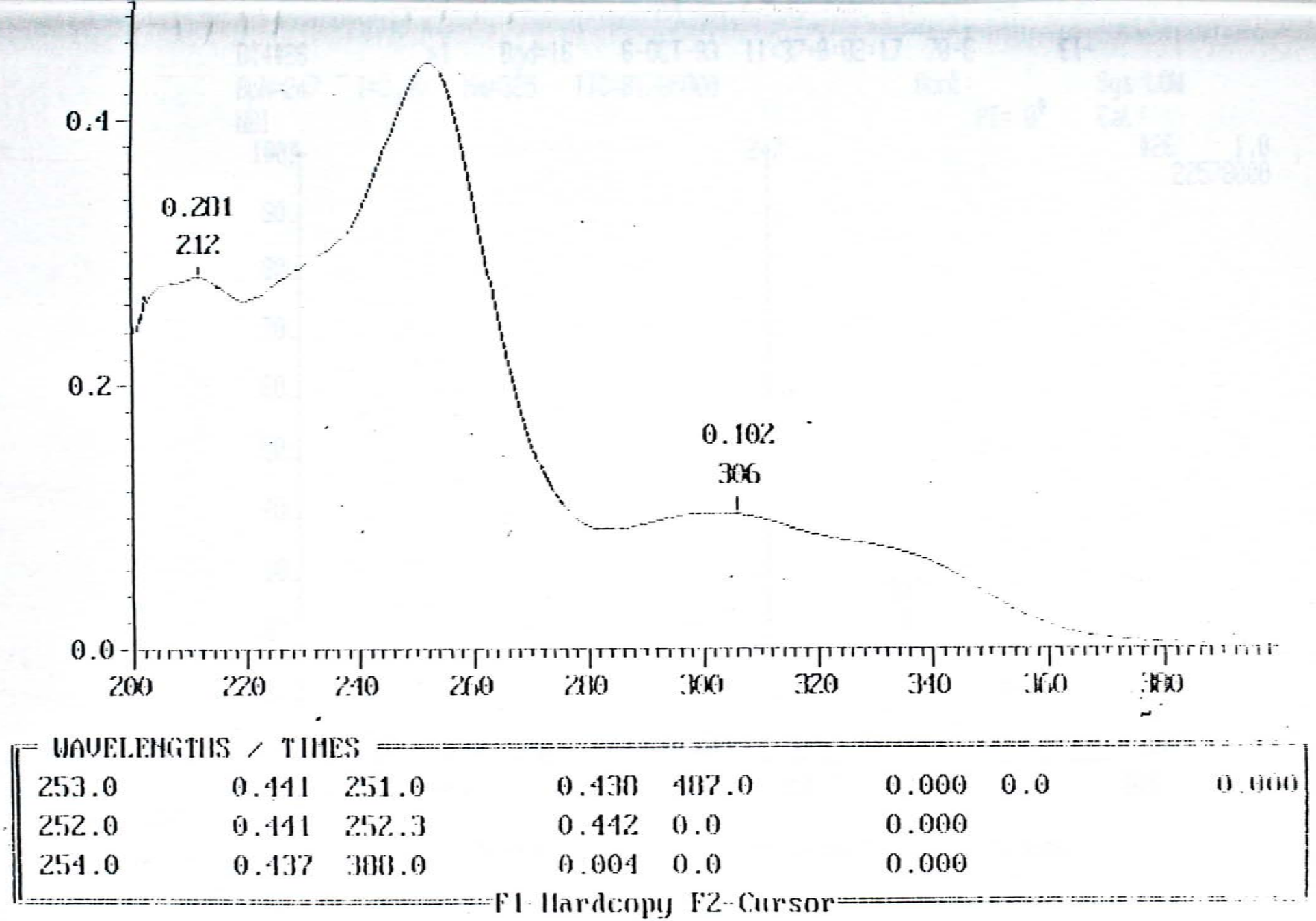


Fig. 4.9. UV spectrum of the inhibitory substance isolated from *Narcissus* bulbs

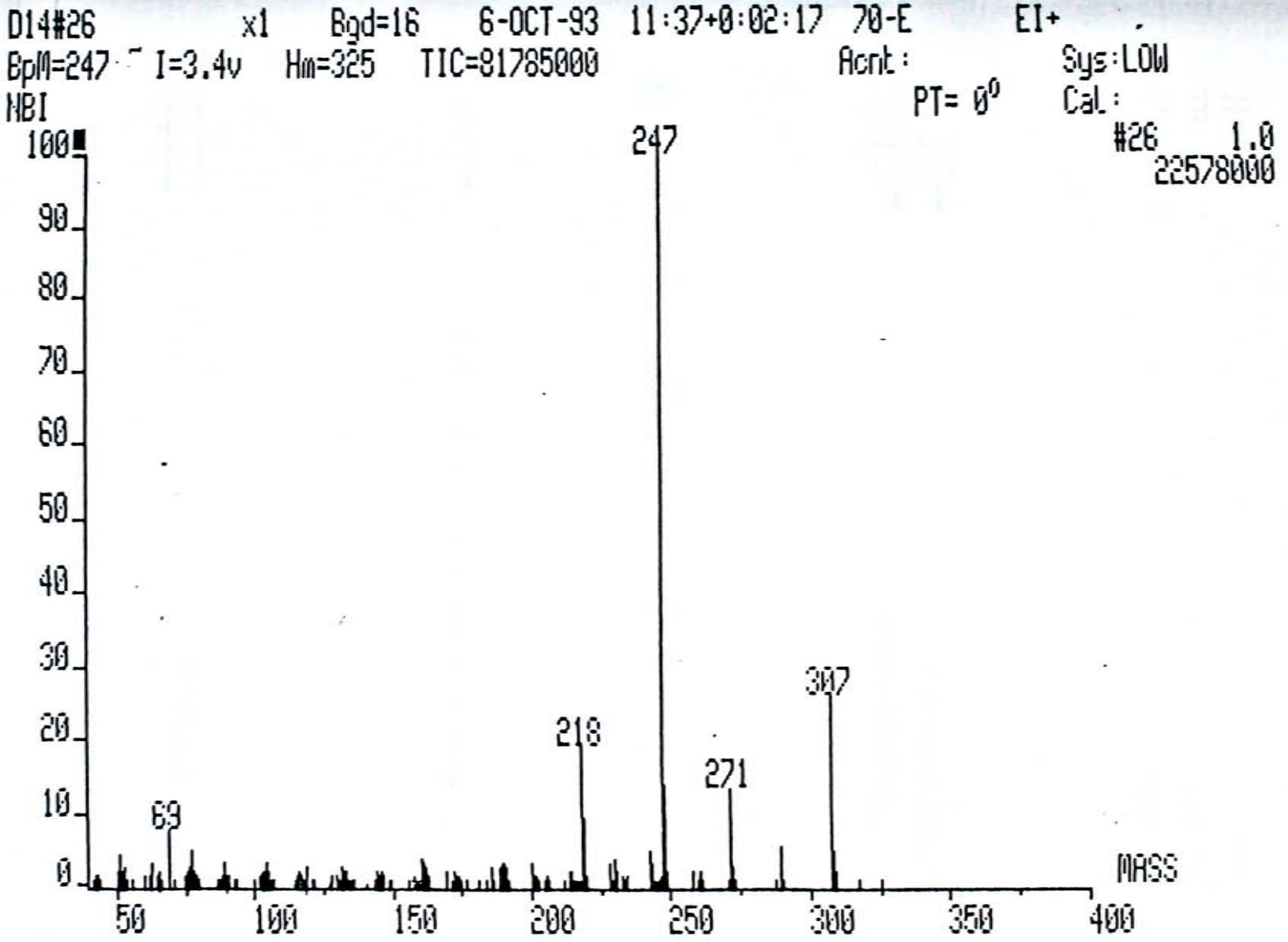


Fig. 4.10. MS spectrum of the substance isolated from *Narcissus* bulbs

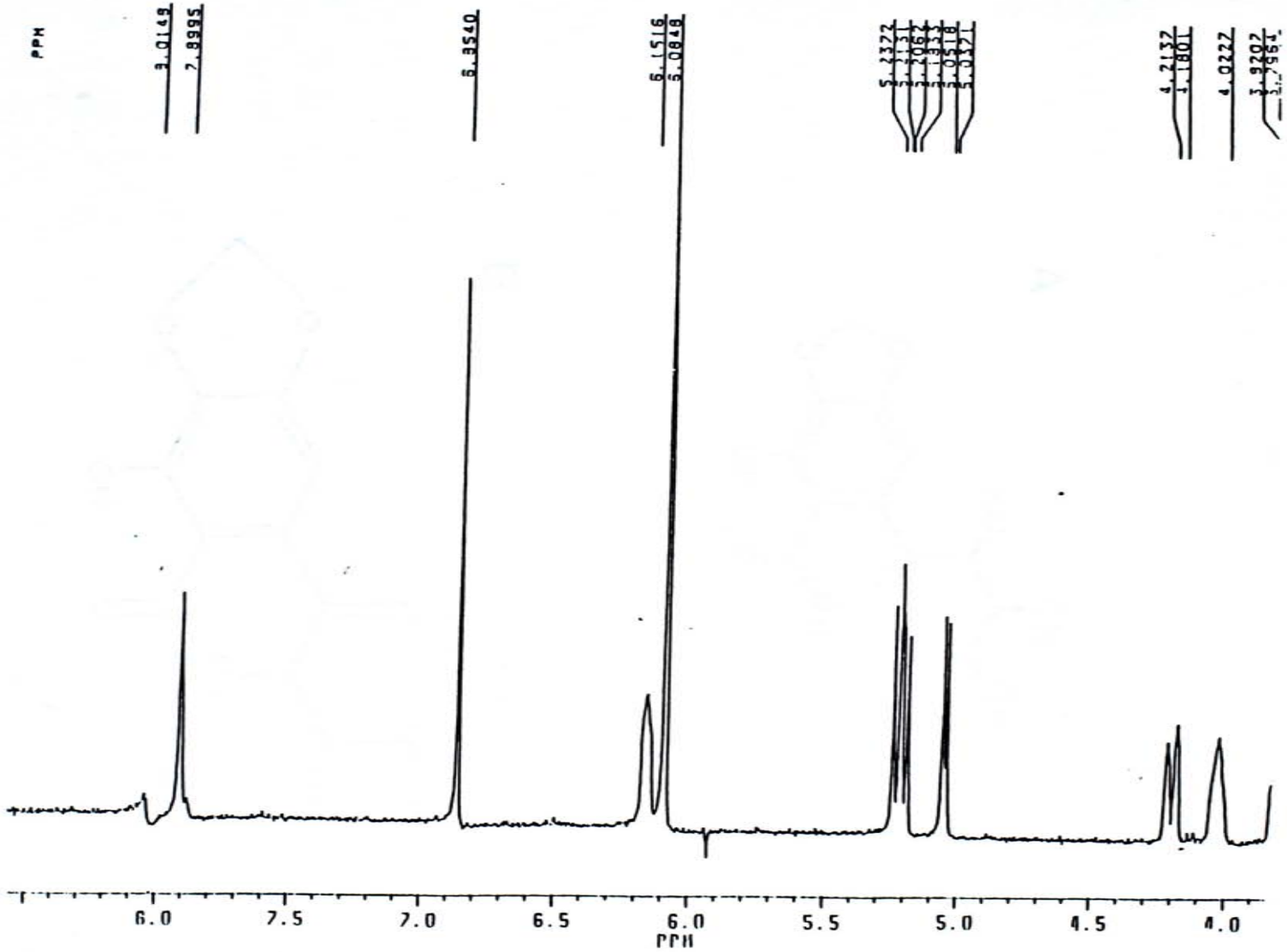


Fig. 4.11. ^{1}HNMR spectrum of the substance isolated from *Narcissus* bulbs

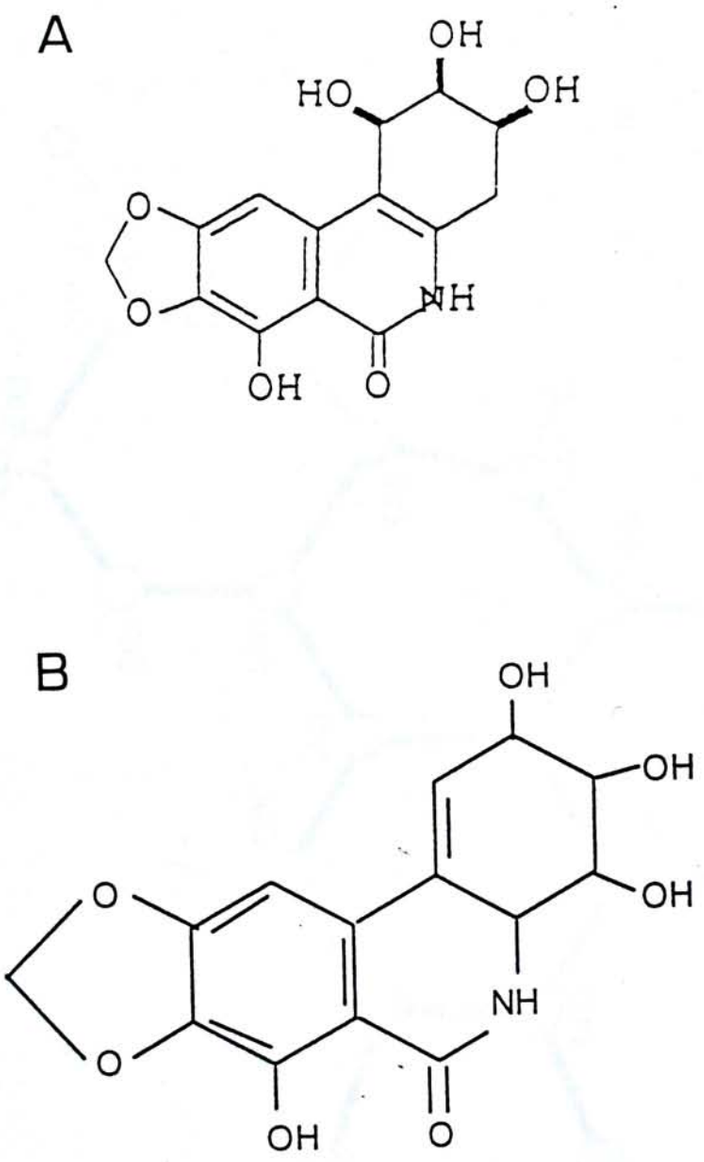


Fig. 4.12. Chemical structure of narciclasine. A, structure originally reported by Piozzi et al. (1968); B, revised structure reported by Mondon and Krohn (1970).

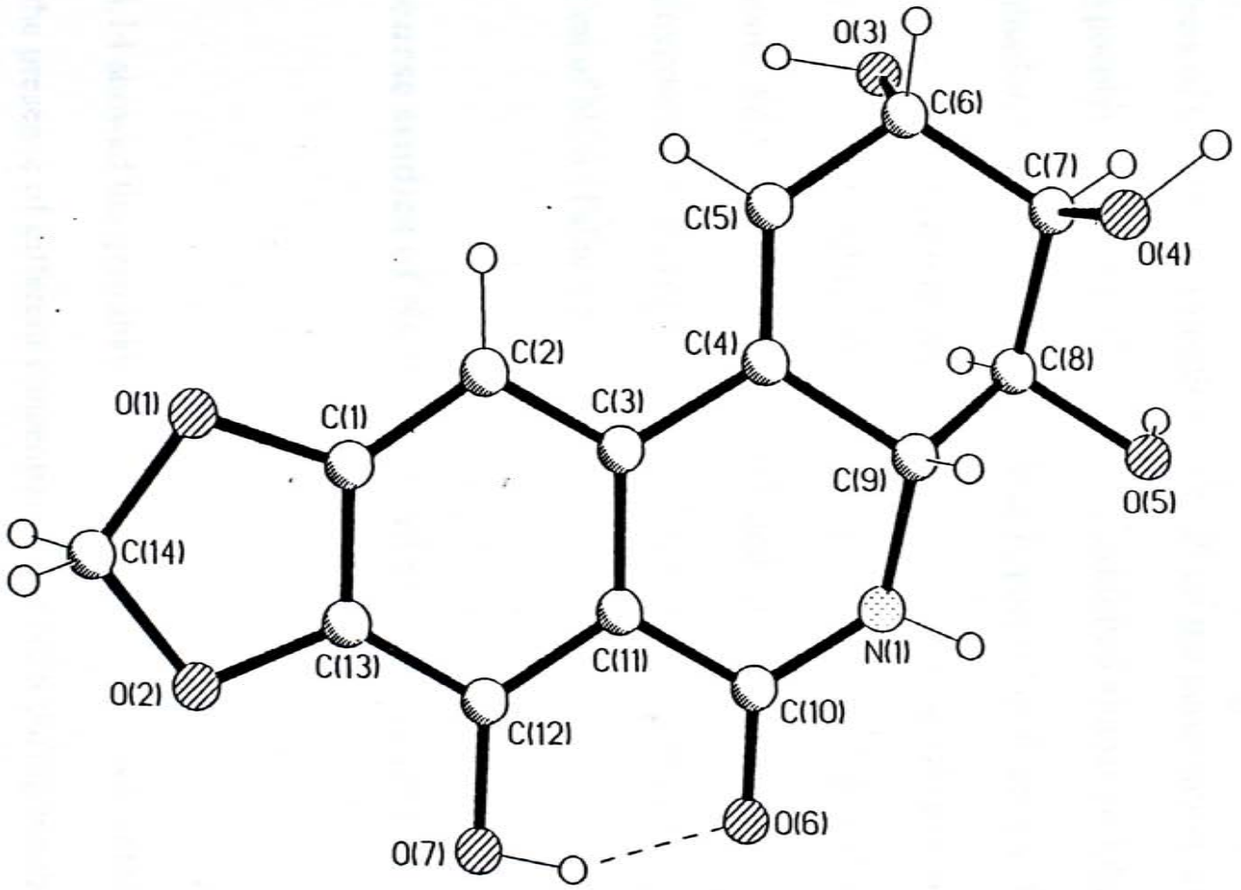


Fig. 4.13. Molecular structure of narciclasine isolated from *Narcissus* bulbs

4.2. Physiological and biochemical studies of NCS

I. Effects of NCS on seed germination and seedling growth of *Brassica*

NCS, after crystallization, was dissolved in methanol. Table 4.6 showed the inhibitory effects of various concentrations of NCS on the germination and the growth of radical and hypocotyl of *Brassica*. NCS (10^{-8}M) exhibited almost no inhibitory effect on the seed germination and elongation of radicle or hypocotyl of *Brassica*. The inhibition to seed germination of *Brassica* was about 82.6% at 10^{-5}M concentration of NCS after 72 hr incubation in dark at 22±2°C. The elongation of radicle of *Brassica* seedlings was suppressed about 86.1% and 92.4% at 10^{-6} and 10^{-5}M concentration of NCS. The elongation of hypocotyl of *Brassica* seedlings, however, was completely inhibited at 10^{-6} M concentration of NCS (Table 4.6).

II. Time course studies of NCS on germination and growth of radish seeds

Fig. 4.14 showed the germination of radish seeds in distilled water (control seeds) or in the presence of different concentrations of NCS during incubation for 60 hr at 22±2°C. NCS had a strong inhibitory effect on germination of radish

Table 4.6 Effect of NCS concentrations on the germination and the elongation of radicle and hypocotyl of germinating *Brassica* seeds

Concentration of NCS (M)	Germination		Length of radicle		Length of hypocotyl	
	No.	%Ctrl	mm	%Ctrl	mm	%Ctrl
0	11.5	100.0	15.8	100.0	7.0	100.0
10^{-8}	12.5	108.7	20.0	126.6	6.7	95.7
10^{-7}	9.7	84.3	14.9	94.3	5.0	71.4
10^{-6}	8.7	75.7	2.2	13.9	0.0	0.0
10^{-5}	2.0	17.4	1.2	7.6	0.0	0.0

Twenty *Brassica* seeds were allowed to germinate in distilled water or different concentrations of NCS in dark for 72 hr at 22±2°C. The number of germinating seeds of *Brassica* was recorded when 1mm radicle was visible after incubation for 72 hr in dark.

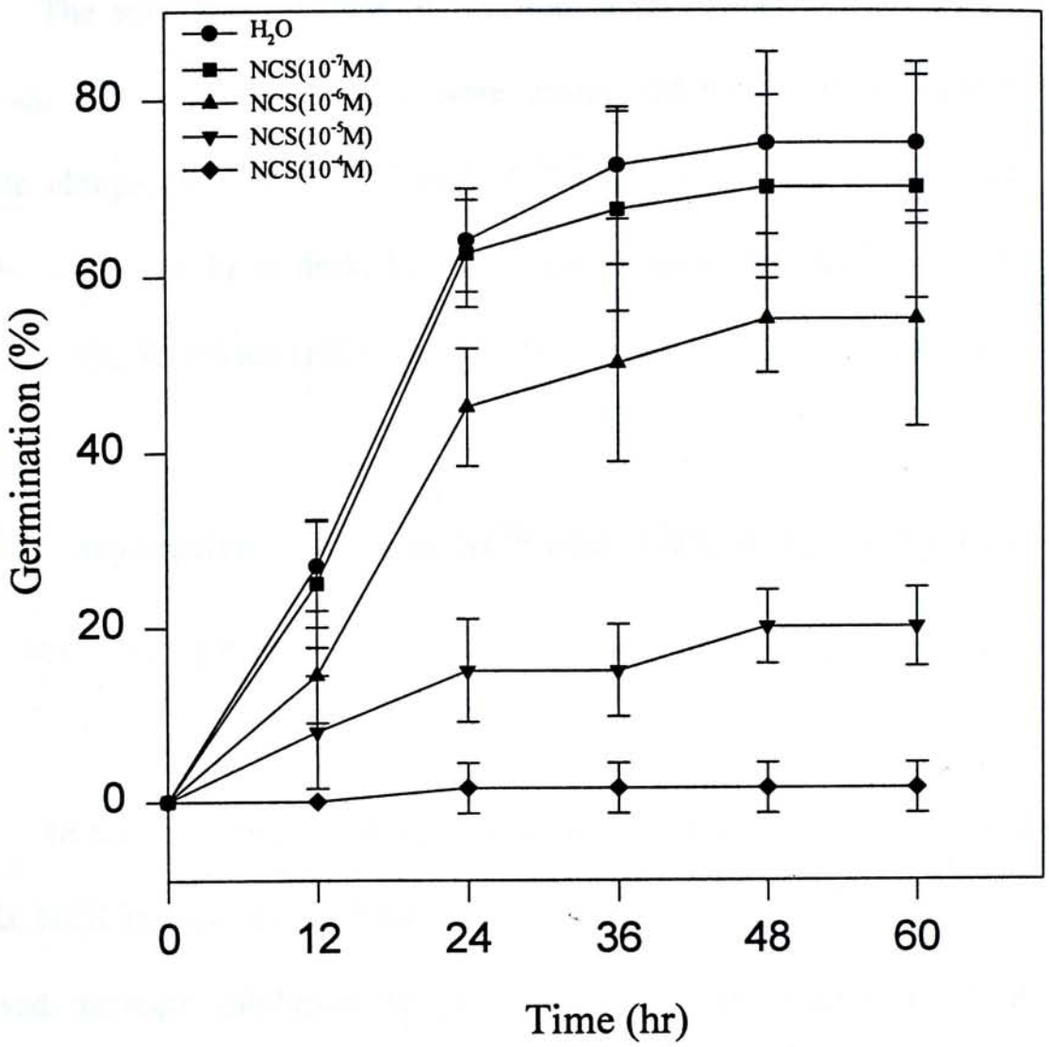


Fig. 4.14 Time course study of NCS on germination of radish seeds. Radish seeds were allowed to germinate in distilled water or various concentrations of NCS as required in dark at 22±2°C. Mean±SD, n=6.

seeds, which increased with increasing concentrations. When NCS concentration increased, the germination rate of radish first started to decrease progressively. In the presence of 10^{-4}M NCS, the germination was totally blocked.

The inhibitory effect of NCS on the radicle elongation of radish seedlings was also shown in Table 4.7. There were around 36.5%, 81.2% and 86.5% inhibitions to radicle elongation at 10^{-7}, 10^{-6} and 10^{-5}M NCS compared to the water control, after incubation for 60 hr in dark. In elongation of hypocotyl, the inhibitions of NCS were about 35.8%, 70.5% and 100% at 10^{-7}, 10^{-6} and 10^{-5}M NCS, respectively (Table 4.8).

III. Comparative studies of NCS and ABA on seeds germination and seedlings growth

In the experiments, comparing to inhibition of ABA to germination of *Brassica* seeds, NCS had the similar inhibitory effect on germination (Table 4.9). However, NCS showed stronger inhibition to germination of seed than that of ABA at 10^{-5}M concentration. There were about 98.3% and 72.4% inhibition to germination of *Brassica* seeds at 10^{-5}M concentration of NCS and ABA. Similar phenomena were observed in rice seeds (Table 4.10). At 10^{-5}M concentration, the inhibitions of NCS and ABA to germination of rice seeds were about 87.8% and 70.3%.

Growth of radicle of *Brassica* seedlings was totally inhibited during its incubation for 72 hr at 10^{-6}M NCS (Table 4.11). ABA (10^{-6}M) showed about 29.9%

Table 4.7 Effect of NCS concentrations on the radical elongation of radish seeds.

NCS (M)	Hours after germination		
	36 hr	48 hr	60 hr
	Length of radical (mm)		
0	5.1± 1.2	12.6±1.6	20.8±0.2
10^{-7}	4.4±1.7	10.8±3.2	13.2±0.4
10^{-6}	3.4±0.4	3.7±0.4	3.9±0.4
10^{-5}	2.5±0.3	2.7±0.3	2.8±0.1

Twenty radish seeds were incubated in a 3.3cm Petri dish with a layer of filter paper containing 1ml different concentrations (10^{-7} - 10^{-5}M) of NCS. Radicle length of radish seedlings were measured up to 60 hr incubation in dark at 22±2°C. Mean ± SD, n=6.

Table 4.8 Effect of NCS concentrations on the hypocotyl elongation of radish seeds.

NCS (M)	Hours after germination		
	36 hr	48 hr	60 hr
	Length of hypocotyl (mm)		
0	1.7±0.6	4.5±0.1	9.5±2.3
10^{-7}	1.6±1.3	3.4±0.4	6.1±1.9
10^{-6}	0.9±0.6	1.4±0.6	2.8±0.5
10^{-5}	0.0	0.0	0.0

The incubation condition of radish seed was the same as in Table 4.7. Length of radish hypocotyls were measured up to 60 hr incubation in dark at 22±2°C. Mean ± SD, n=6.

Table 4.9 Effects of NCS and ABA concentrations on the percentage of germination of *Brassica* seed.

Time (hr)	Germination (%)						
	NCS (M)				ABA (M)		
	0	10^{-7}	10^{-6}	10^{-5}	10^{-7}	10^{-6}	10^{-5}
24	14.2±8.6	4.2±5.8	2.5±4.2	0.0	10.8±5.8	3.3±4.1	0.8±2.0
36	65.0±8.7	54.2±7.5	43.8±8.5	0.0	58.8±7.5	48.8±6.3	6.3±2.5
48	71.3±4.8	66.3±2.5	58.8±8.5	1.3±2.5	62.5±2.9	55.0±8.2	18.8±4.8
60	72.5±6.5	66.3±2.5	60.0±8.2	1.3±2.5	62.5±2.9	56.3±10.3	20.0±7.1
72	72.5±6.5	66.3±2.5	60.0±8.2	1.3±2.5	62.5±2.9	56.3±10.3	20.0±7.1

Twenty *Brassica* seeds were allowed to germination in a 3.3cm Petri dish containing various concentrations of NCS or ABA in dark up to 72 hr. The seeds were recorded as germinated when 1mm radicle was visible. Mean ± SD, n=6.

Table 4.10 Effects of NCS and ABA concentrations on the germination of rice seeds.

Treatment	Concentration (M)	Germination (%)
H_2O	0	90.0±10.0
NCS	10^{-7}	96.7±5.8
NCS	10^{-6}	80.0±4.6
NCS	10^{-5}	20.0±10.0
ABA	10^{-7}	88.3±9.8
ABA	10^{-6}	83.3+5.8
ABA	10^{-5}	26.7±11.6

Ten rice seeds were allowed to germinate in a 3.3cm Petri dish with a layer of filter paper containing different concentrations of NCS or ABA for 72 hr in dark. The seed were recorded as germinated when 1mm radicle or 1mm coleoptile was visible. Mean ± SD, n=6.

Table 4.11 Effects of NCS and ABA concentrations on the radicle elongation of *Brassica* seedlings.

Time (hr)	Length of radicle (mm)						
	NCS (M)				ABA (M)		
	0	10^{-7}	10^{-6}	10^{-5}	10^{-7}	10^{-6}	10^{-5}
48	12.4±2.1	7.8±2.1	2.1±0.1	0	17.4±2.1	14.4±0.4	4.4±1.4
60	18.4±0.6	15.0±2.5	2.3±0.1	0	19.4±0.1	18.1±3.1	7.6±2.5
72	38.1±2.0	29.1±2.9	2.5±0.4	0	32.8±1.5	26.7±0.3	9.3±0.8

Brassica seeds were allowed to germinate at the same condition as in Table 4.9. Radicle length of *Brassica* seedlings were measured from 48 hr to 72 hr after incubation in dark. Mean ± SD, n=6.

inhibition to the radicle growth of *Brassica* seedlings. Growth of hypocotyl of *Brassica* seedlings was practically blocked by 10^{-6}M NCS (Table 4.12). The inhibition by ABA was only about 23.4% at 10^{-6}M concentration. In the growth of radicles of rice seedlings (Table 4.13), inhibitions of NCS and ABA at 10^{-6}M concentration were 62.7% and 24.2%, respectively, after 72 hr incubation in dark. The growth of coleoptile of rice seedlings (Table 4.14) exhibited a similar effect in a range of concentrations of 10^{-7} - 10^{-6} M NCS. ABA (10^{-6}M) was 34.2% inhibition to the growth of coleoptile of rice seedlings. NCS and ABA at 10^{-5}M concentration, however, showed differetial action on elongation of radicle and coleoptile of rice seedlings. The radicle elongation of rice seedlings was totally inhibited by 10^{-5}M NCS (Table 4.13), but the coleoptile of rice seedlings was totally inhibited by 10^{-5}M ABA (Table 4.14).

IV. Interaction between NCS and phytohormones

A. Interaction of NCS with ABA

Table 4.15 showed the effect of NCS and ABA on seed germination of *Brassica.* After incubation for 72 hr in dark at 22±2°C, there were about 66.3%, 67.5% and 60.0% germination of *Brassica* seeds which were treated with 10^{-7}M, $5x10^{-7}$M and 10^{-6}M concentrations of NCS, respectively. However, in the simultaneous presence of 10^{-6}M ABA, there were about 66.5%, 68.5% and 60.0% germination of *Brassica* seeds which were treated with 10^{-7}M , $5x10^{-7}$M and 10^{-6}M NCS, It appears that the inhibitory effect of ABA and NCS was not additive. ABA seems less effective in the early stage and more effective in the late stage of germination. It might probably be due to different rate of uptake of these two compounds. The non-additive inhibitory effect of ABA and NCS was

Table 4.12 Effects of NCS and ABA concentrations on the hypocotyl elongation of *Brassica* seedlings.

Time (hr)	Length of hypocotyl (mm)						
	NCS (M)				ABA (M)		
	0	10^{-7}	10^{-6}	10^{-5}	10^{-7}	10^{-6}	10^{-5}
48	2.8±0.7	1.6±0.2	0.6±0.1	0	3.1±0.3	2.4±0.4	1.1±0.2
60	6.9±0.4	4.5±1.0	0.7±0.1	0	5.1±0.4	5.1±0.4	2.3±0.4
72	11.1±0.1	9.9±0.1	0.7±0.3	0	9.7±0.6	8.5±1.7	3.2±1.2

Brassica seeds were allowed to germinate at the same condition as in Table 4.9. Hypocotyl length of *Brassica* seedlings were measured from 48 hr to 72 hr after incubation in dark. Mean± SD, n=6.

Table 4.13 Effects of NCS and ABA concentrations on the radical elongation of rice seedlings.

Treatment	Concentration (M)	Length of radical (mm)
H_2O	0	15.3±3.2
NCS	10^{-7}	14.7±1.4
NCS	10^{-6}	5.7±1.1
NCS	10^{-5}	0.0±0.4
ABA	10^{-7}	12.2±2.1
ABA	10^{-6}	11.6±1.1
ABA	10^{-5}	5.0±1.4

Rice seeds were incubated at the same condition as in Table 4.10. Elongation of radicle of rice were measured after 72 hr incubation in dark. Mean ± SD, n=6.

Table 4.14 Effects of NCS and ABA concentrations on the coleoptile elongation of rice seedlings.

Treatment	Concentration (M)	Length of coleoptile (mm)
H_2O	0	3.8±0.3
NCS	10^{-7}	3.7±0.2
NCS	10^{-6}	2.7±0.3
NCS	10^{-5}	1.8±0.6
ABA	10^{-7}	2.8±0.7
ABA	10^{-6}	2.5±0.3
ABA	10^{-5}	0.0

Rice seeds were incubated at the same condition as described in Table 4.10. Elongation of coleoptiles of rice were measured after 72 hr incubation in dark. Mean ± SD, n=6.

Table 4.15 Effects of NCS and ABA on germination of *Brassica* seeds.

Treatment	Percentage of Germination				
	24 hr	36 hr	48 hr	60 hr	72 hr
Water	14.2±8.6	65.0±8.7	71.3±4.8	72.5±6.5	72.5±6.5
NCS (10^{-7}M)	4.2±5.8	54.2±7.5	66.3±2.5	66.3±2.5	66.3±2.5
NCS ($5x10^{-7}$M)	6.0±5.2	56.0±7.1	67.5±5.8	67.5±5.8	67.5±5.8
NCS (10^{-6}M)	2.5±4.2	43.8±8.5	58.8±8.5	60.0±8.2	60.0±8.2
ABA (10^{-7}M)	10.8±5.8	58.8±7.5	62.5±2.9	62.5±2.9	62.5±2.9
ABA (10^{-6}M)	3.3±4.1	48.8±6.3	55.0±8.2	56.3±10.3	56.3±10.3
ABA (10^{-6}M) + NCS (10^{-7}M)	5.8±4.2	51.7±10.8	55.8±8.6	55.8±8.6	55.8±8.6
NCS ($5x10^{-7}$M)	6.8±5.5	50.1±17.0	55.0±16.6	55.0±16.6	55.0±16.6
NCS (10^{-6}M)	1.7±2.6	34.2±12.0	45.0±17.9	45.0±17.9	45.0±17.9

Twenty *Brassica* seeds were allowed to germinate in a 3.3cm Petri dish with a layer filter paper containing various concentrations of NCS, ABA or both of them up to 72 hr in dark. The seeds were recorded as germinated when 1mm radicle was visible. Mean±SD , n=6.

also observed in the elongation of radicle and hypocotyl of *Brassica* seedlings (Tables 4.16 and 4.17). NCS was more effective on the elongation of radicle and hypocotyl than that of ABA.

B. Interaction of NCS with IAA

The influence of NCS on IAA action was examined by wheat coleoptile section test. The results showed that 10^{-6}M IAA promoted elongation of coleoptile section of wheat when the coleoptile section of control -from 5.0mm to 7.8mm was compared with the IAA-treated coleoptile section-from 5.0mm to 8.6mm (Table 4.18). NCS inhibited the elongation of coleoptile section at 10^{-6}M and 10^{-5}M. While the coleoptiles were incubated in both NCS and IAA, the higher concentrations of IAA (10^{-6} to 10^{-5}M) could partially reverse the inhibitory effects of 10^{-7}M NCS, but IAA could not reverse the inhibition of NCS at 10^{-6} and 10^{-5}M concentrations.

C. Interaction of NCS with gibberellin

The effects of NCS on gibberellin activity was tested using the barley endosperm bioassay. Table 4.19 showed that GA_3 (10^{-8}-10^{-6}M) could markedly stimulate α-amylase formation. The promoting activity of 10^{-6}M GA_3 was from 6.0μg glucose/seed (control) to 41.4μg glucose/seed. NCS in the range of concentrations tested significantly reduced

Table 4.16 Effects of interaction of NCS and ABA on radicle elongation of *Brassica* seeds.

Treatment	Length of Radical (mm)		
	48 hr	60 hr	72 hr
Water	13.9±2.1	19.8±0.6	38.1±2.0
NCS (10^{-7}M)	7.8±2.1	15.0±2.5	29.1±2.9
NCS ($5x10^{-7}$M)	3.2±0.6	3.6±0.2	5.4±2.1
NCS (10^{-6}M)	2.1±0.1	2.3±0.1	2.5±0.4
ABA (10^{-7}M)	15.9±2.1	19.4±0.1	32.8±1.5
ABA (10^{-6}M)	14.4±0.4	18.1±3.1	26.7±0.3
ABA (10^{-6}M) + NCS (10^{-7}M)	12.0±0.4	21.1±1.3	29.8±1.0
NCS ($5x10^{-7}$M)	2.7±0.4	3.8±0.1	5.3±0.6
NCS (10^{-6}M)	2.0±0.1	2.4±0.1	2.6±0.1

Brassica seeds were allowed to germinate at the same condition as described in Table 4.15. Radicle length were measured from 48 hr to 72 hr. Mean±SD, n=6.

Table 4.17 Effects of interaction of NCS and ABA on the hypocotyl elongation of *Brassica* seeds.

Treatment	Length of Hypocotyl (mm)		
	48 hr	60 hr	72 hr
Water	2.8±0.7	6.9±0.4	11.1±0.1
NCS (10^{-7}M)	1.7±0.2	4.5±1.0	9.9±0.1
NCS ($5x10^{-7}$M)	1.5±1.5	1.7±0.1	2.2±0.7
NCS (10^{-6}M)	0.6±0.1	0.7±0.1	0.7±0.3
ABA (10^{-7}M)	3.1±0.3	5.1±0.4	9.7±0.6
ABA (10^{-6}M)	2.4±0.4	5.1±0.4	8.5±1.7
ABA (10^{-6}M) +			
NCS (10^{-7}M)	1.9±0.7	4.8±0.4	8.8±0.4
NCS ($5x10^{-7}$M)	1.7±0.4	1.9±0.1	2.6±0.6
NCS (10^{-6}M)	0.6±0.4	0.9±0.1	1.0±0.2

Brassica seeds were allowed to germinate at the same condition as described in Table 4.15. Hypocotyl lengths were measured from 48 hr to 72 hr. Mean±SD, n=6.

Table 4.18 Effects of different concentrations of IAA and NCS on the elongation of wheat coleoptiles

IAA (M)	Length of coleoptile (mm)			
	0	NCS (10^{-7}M)	NCS (10^{-6}M)	NCS (10^{-5}M)
0	7.8±0.6	7.2±0.5	6.8±0.4	6.1±0.1
10^{-7}	7.9±0.5	7.6±0.5	6.9±0.4	6.1±0.1
10^{-6}	8.6±0.5	8.2±0.6	7.3±0.6	6.1±0.1
10^{-5}	8.3±0.3	8.1±0.4	7.0±0.4	6.1±0.1

Five mm sections of coleoptile of wheat were incubated in 1% sucrose phosphate-citrate solution (pH5.4) containing various concentration of NCS , IAA or both of them for 20 hr in darkness at 28°C, and the length of coleoptile sections was measured. Each value was the average of 10 (one sample) ±SD, n=6.

Table 4.19 Effects of different concentrations of GA_3 and NCS on α-amylase activities (μg glucose/seed/hr) in barley seeds.

	α-amylase activity (μg glucose / seed/hr)			
	NCS (M)			
GA_3 (M)	0	10^{-7}	10^{-6}	10^{-5}
0	6.0±0.5	5.6±0.9	5.7±0.1	4.4±0.3
10^{-8}	31.4±1.8	28.2±5.5	12.0±3.0	5.6±0.6
10^{-7}	39.2±2.4	37.1±3.8	21.8±2.6	6.7±1.9
10^{-6}	41.4±4.1	41.0±3.9	29.0±2.9	7.7±0.6

α-amylase activity was expressed as μg glucose released per seed per hr. Each treatment is the mean of six replicates. Each replicate comprised of four embryo-free barley endosperms.

α-amylase formation in this bioassay. When incubated concurrently with GA_3, NCS (10^{-7} M) had no inhibitory effect on GA activity. But the GA-induced α-amylase activity was totally blocked by 10^{-5}M NCS.

D. Interaction of NCS with cytokinin

Table 4.20 and Table 4.21 showed the effects of NCS on cytokinin activity which were tested by the radish cotyledon expansion. The maximum promotion of expansion was observed in excised radish cotyledons treated with 10^{-5}M BA after incubated for 48 hr at 28°C either in ligth or in dark. It was found that there were about 216.6% and 204.7% promotion of excised cotyledons expansion. NCS (10^{-6} M) inhibited the effect of BA on excised cotyledons expansion under light or dark and it was approximately 73.4% or 47.7% inhibition. NCS (10^{-5}M and 10^{-4}M) completely inhibited the promotion of BA to excised cotyledons expansion either in light or in dark.

V. Effects of NCS and BA on expansion, chlorophyll content and carotenoid content of excised cotyledons

A. Effects of NCS and BA on chlorophyll and carotenoid content of excised cotyledons

Table 4.20 Effects of NCS and BA on the increase of fresh weight of excised radish cotyledons growing in light after germination.

BA (M)	Δ fresh weight (mg/cotyl.) NCS (M)				
	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
0	7.8±0.3	7.3±0.7	1.9±0.3	0.8±0.1	0.3±0.1
10^{-6}	13.5±0.5	12.7±1.9	2.9±0.1	0.7±0.1	0.4±0.1
10^{-5}	16.9±0.4	16.7±0.2	4.5±0.7	1.1±0.3	0.5±0.1
10^{-4}	15.3±0.8	11.3±1.2	2.9±0.6	1.0±0.2	0.7±0.1

Excised radish cotyledons were obtained from radish seeds germinated for 48 hr in darkness at 22±2°C. Excised cotyledons were incubated under continuous light in various concentration of NCS, BA or both of them for 48 hr at 28°C. The change of fresh weight of excised cotyledons was determined after incubation in light for 48 hr. Each sample included ten excised cotyledons. The average initial fresh weight of excised cotyledon was 6.0-7.0 mg/cotyl. Mean±SD, n=6.

Table 4.21 Effects of NCS and BA on the increase of fresh weight of excised radish cotyledons growing in dark after germination.

BA (M)	Δ fresh weight (mg/cotyl.) NCS (M)				
	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
0	4.3±0.7	3.9±0.2	2.5±0.2	1.1±0.1	0.9±0.1
10^{-6}	6.7±0.2	6.4±0.6	3.4±0.3	1.0±0.1	0.8±0.1
10^{-5}	8.8±0.2	8.6±0.1	4.6±0.6	1.0±0.2	0.8±0.1
10^{-4}	8.5±0.2	6.6±0.4	3.8±0.3	1.1±0.1	0.7±0.1

Excised radish cotyledons were obtained from radish seeds germinated for 48 hr in darkness at 22±2°C. Excised cotyledons were incubated in darkness in various concentration of NCS, BA or both of them for 48 hr at 22±2°C. The change of fresh weight of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. The average initial fresh weight of excised cotyledon was 6.0-7.0mg/cotyl. Mean±SD, n=6.

Table 4.22 and Table 4.23 showed the effect of NCS and BA on the chlorophyll content or carotenoid content of excised radish cotyledon. BA (10^{-5}M) exhibited the greatest increase in chlorophyll content or carotenoid content in excised cotyledon after incubation for 48 hr in light or in dark at 28°C. Total chlorophyll content in 10^{-5} M BA treated cotyledons was about 125.5% of the control (Table 4.22). The greening of cotyledons after exposing to light, however, decreased drastically depending on NCS concentrations. Higher concentration of NCS (10^{-5}M) led to completely block to greening of excised radish cotyledons in light. Both chlorophyll a and chlorophyll b contents were affected by NCS. And Chl a appeared to have more sensitivity to NCS. As shown in Table 4.23, carotenoid content was approximately doubled by 10^{-5}M BA after incubation for 48hr in dark and NCS reduced the amount of carotenoid. The carotenoid content was reduced by NCS, which was about 33.0%, 81.8% and 89.8% at 10^{-7}M, 10^{-6}M and 10^{-5}M, respectively. When cotyledons were incubated simultaneously with NCS(10^{-7} -10^{-4}M) and BA (10^{-6} -10^{-4}M) respectively, the inhibition of 10^{-7}M NCS could be reversed by BA (10^{-6} -10^{-4}M). The inhibition of 10^{-6} -10^{-4}M concentrations of NCS to carotenoid content was hardly reversed by BA, even in the presence of 10^{-5}M BA.

Fig. 4.15 and Fig. 4.16 showed the time course studies on excised radish cotyledons growth up to 60hr in light at 28°C. NCS had inhibitory effects on the excised cotyledons growth from 12hr to 60hr in light. Growth of excised radish cotyledons was completely blocked by 10^{-5}M NCS both in light and in dark. At 10^{-6}M NCS, growth of excised cotyledons was reduced about 79.1% or 61.0% comparing to that of control after

Table 4.22 Effect of NCS and BA on the chlorophyll contents of excised radish cotyledons under light after germination.

BA (M)	Chlorophyll content (µg/cotyl.)								
	0			NCS (10^{-7})			NCS (10^{-6})		
	chl a	chl b	total	chl a	chl b	total	chl a	chl b	total
0	11.4±0.90	4.5±0.02	16.1±0.87	8.2±0.24	0.9±0.33	9.3±0.57	0.32±0.45	0.14±0.28	0.46±0.75
10^{-6}	13.1±1.11	3.5±0.80	17.0±1.93	12.7±1.01	3.3±0.59	16.2±1.63	0.27±0.71	0.11±0.35	0.39±1.06
10^{-5}	15.3±0.52	4.6±0.57	20.2±1.11	12.9±1.04	3.7±0.17	16.7±1.23	0.31±0.21	0.18±0.02	0.51±0.26
10^{-4}	14.1±0.68	3.7±0.47	17.9±1.16	12.6±0.19	3.2±0.12	15.9±0.07	0.32±0.17	0.17±0.17	0.49±0.31

Excised radish cotyledons were obtained from radish seeds germinated for 48 hr in light at 22±2°C. Excised cotyledons were incubated in darkness in various concentration of NCS, BA or both of them for 48 hr at 22±2°C. The chlorophyll content of excised cotyledons was determined after incubation for 48 hr. Control was incubated in distilled water. Each sample included ten excised cotyledons. Mean±SD, n=6.

Table 4.23 Effects of NCS and BA on carotenoid contents (μg/cotyl) of excised radish cotyledons for 48 hr in dark after germination.

	Carotenoid content (μg/cotyl.)				
	NCS (M)				
BA (M)	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
0	0.88±0.17	0.59±0.02	0.16±0.03	0.09±0.02	0.08±0.03
10^{-6}	1.35±0.11	1.18±0.03	0.22±0.03	0.10±0.02	0.09±0.01
10^{-5}	1.70±0.07	1.23±0.19	0.28±0.07	0.11±0.01	0.10±0.02
10^{-4}	1.41±0.02	0.95±0.02	0.23±0.02	0.11±0.01	0.09±0.03

Excised radish cotyledons were obtained from radish seeds germinated for 48 hr in darkness at 22±2°C. Excised cotyledons were incubated in darkness in various concentration of NCS, BA or both of them for 48 hr at 22±2°C. The carotenoid content of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. Mean±SD, n=6.

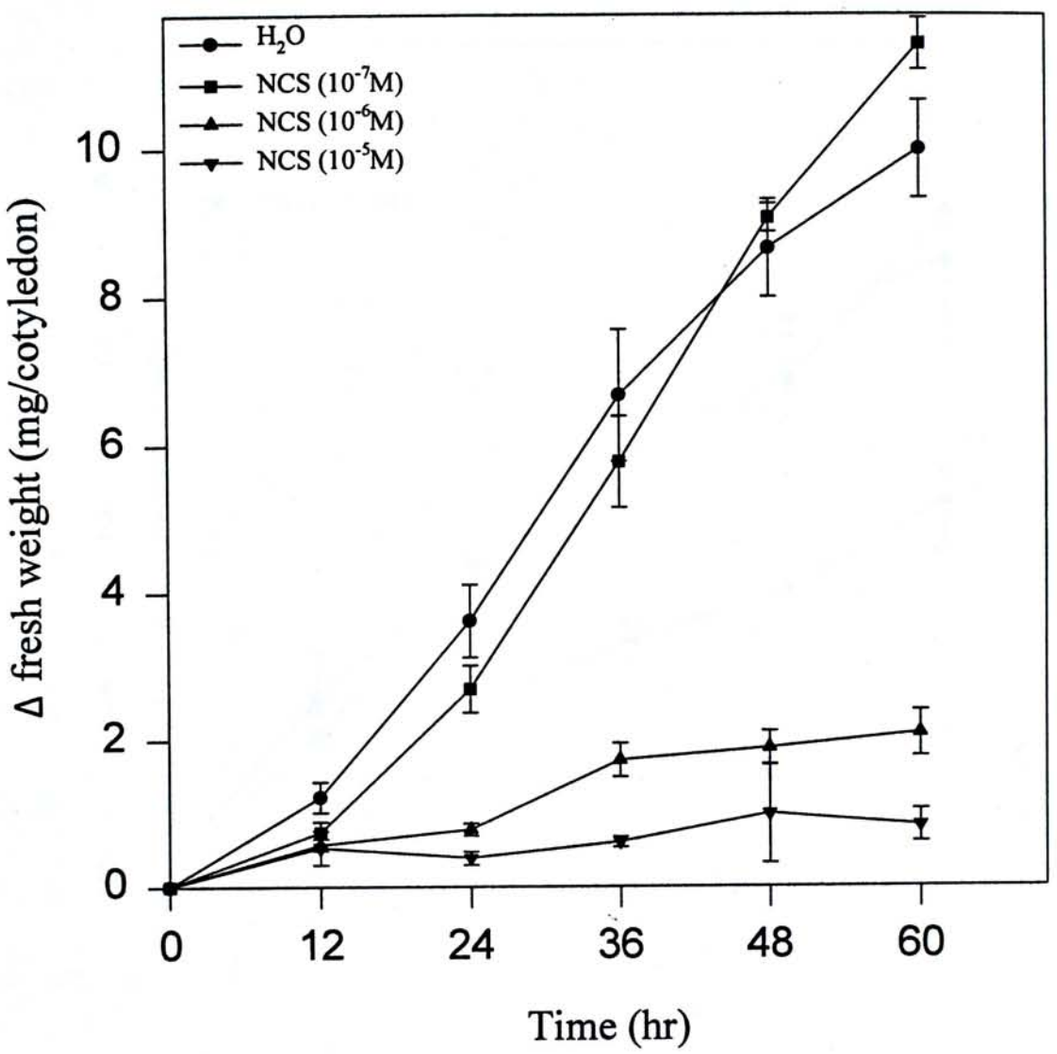


Fig. 4.15 Time course study on the increase in fresh weight of excised cotyledons incubated in different concentrations of NCS in light. Excised cotyledons were obtained from radish seeds germination for 48 hr in dark at 22±2°C. The average initial fresh weight of excised cotyledon was 6.0-7.0mg/cotyledon. Mean±SD, n=6.

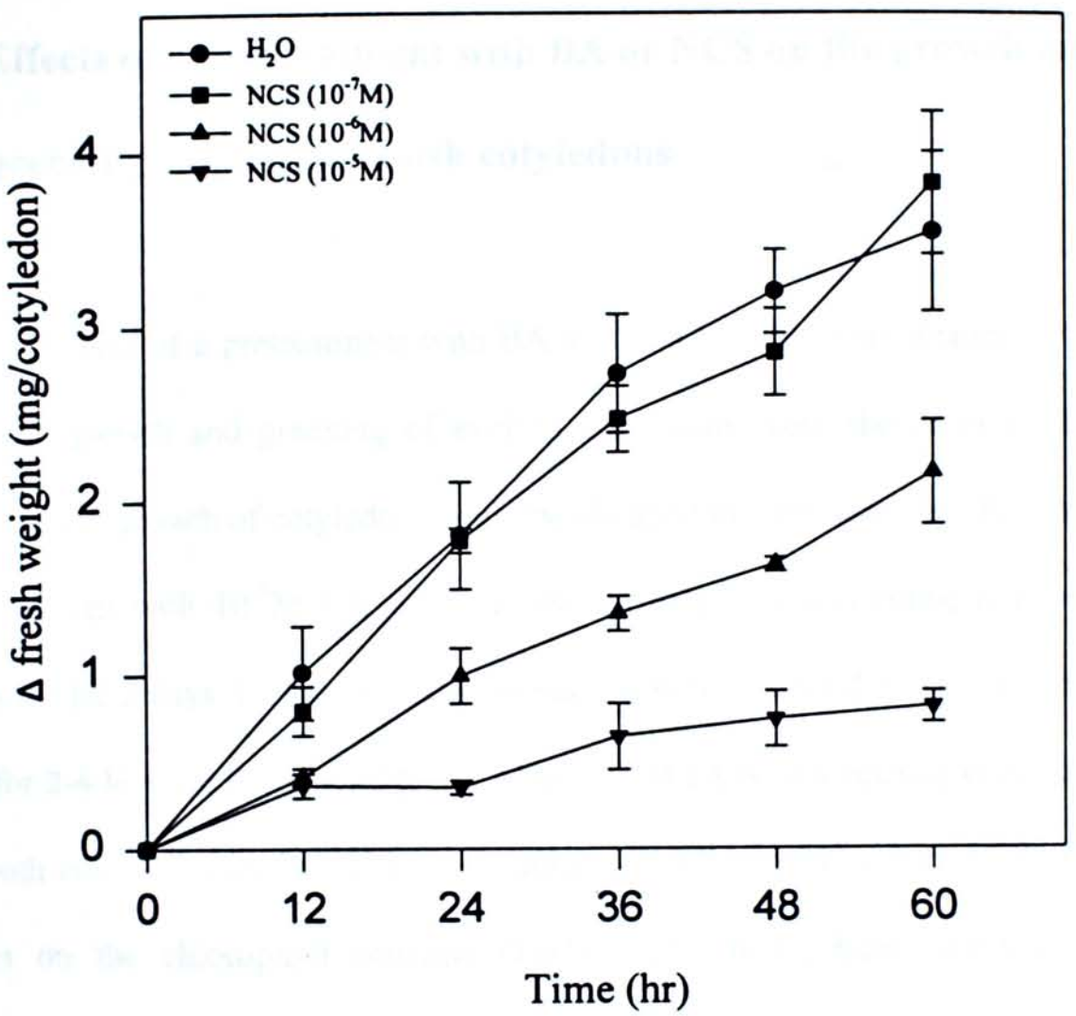


Fig. 4.16 Time course study of NCS on the growth of excised cotyledons in dark. Excised radish cotyledons, from germination of radish seeds for 48 hr in dark at 22±2°C, were incubated in various concentrations in dark of NCS. Change of fresh weight of excised cotyledons was determined during incubation in light for 48 hr. The average initial fresh weight of excised cotyledon was 6.0 -7.0mg/ cotyledon. Mean±SD, n=6.

incubation for 60hr in light or in dark, while a 10^{-7}M concentration of NCS mostly had no inhibitory effect either in light or in dark. There was complete inhibition to chlorophyll synthesis at 10^{-6}M NCS during a period of growth for 60 hr (Fig. 4.17). In the carotenoid content also completely inhibited by 10^{-6}M NCS (Fig. 4.18).

B. Effects of a pretreatment with BA or NCS on the growth and greening of excised radish cotyledons

Effects of a pretreatment with BA and of a simultaneous treatment with BA and NCS on growth and greening of excised cotyledons were shown in Table 4.24. The responses in growth of cotyledons could be obtained in a pretreatment with BA for 5 min. Pretreatment with 10^{-5}M BA for 4 hr was as active as a continuous exposure to the hormone for 2 days. However, when the excised radish cotyledons pretreated with 10^{-5} M BA for 2-4 hr were transferred to 10^{-6}M or 10^{-5} M NCS, BA activity completely blocked by both concentrations of NCS. Pretreatment with 10^{-5} M BA did not show significant effect on the chlorophyll contents (Table 4.25). When those cotyledons with BA pretreated for different times were transferred to either in 10^{-6}M or in 10^{-5}M NCS, chlorophyll formation was completely repressed.

Reversibly, effects of pretreatment with different concentrations of NCS for different times on growth and greening of excised radish cotyledons were also studied.

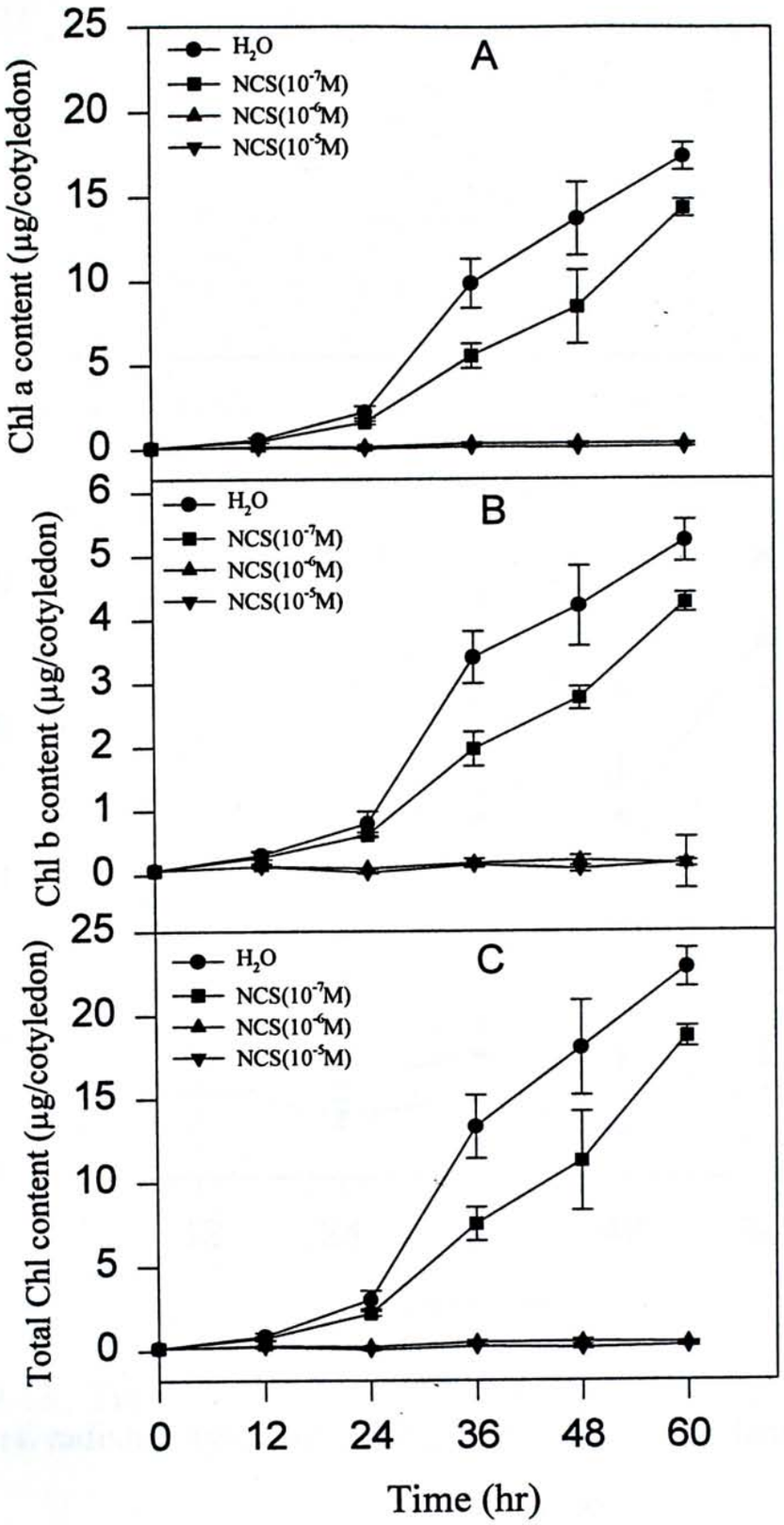


Fig. 4.17 Time course study on the increase of chlorophyll content of excised cotyledons incubated in different concentrations of NCS in light. Excised cotyledons were obtained as in Fig. 4.15.
A: chl a; B: chl b; C: total chl. Mean±SD, n=6.

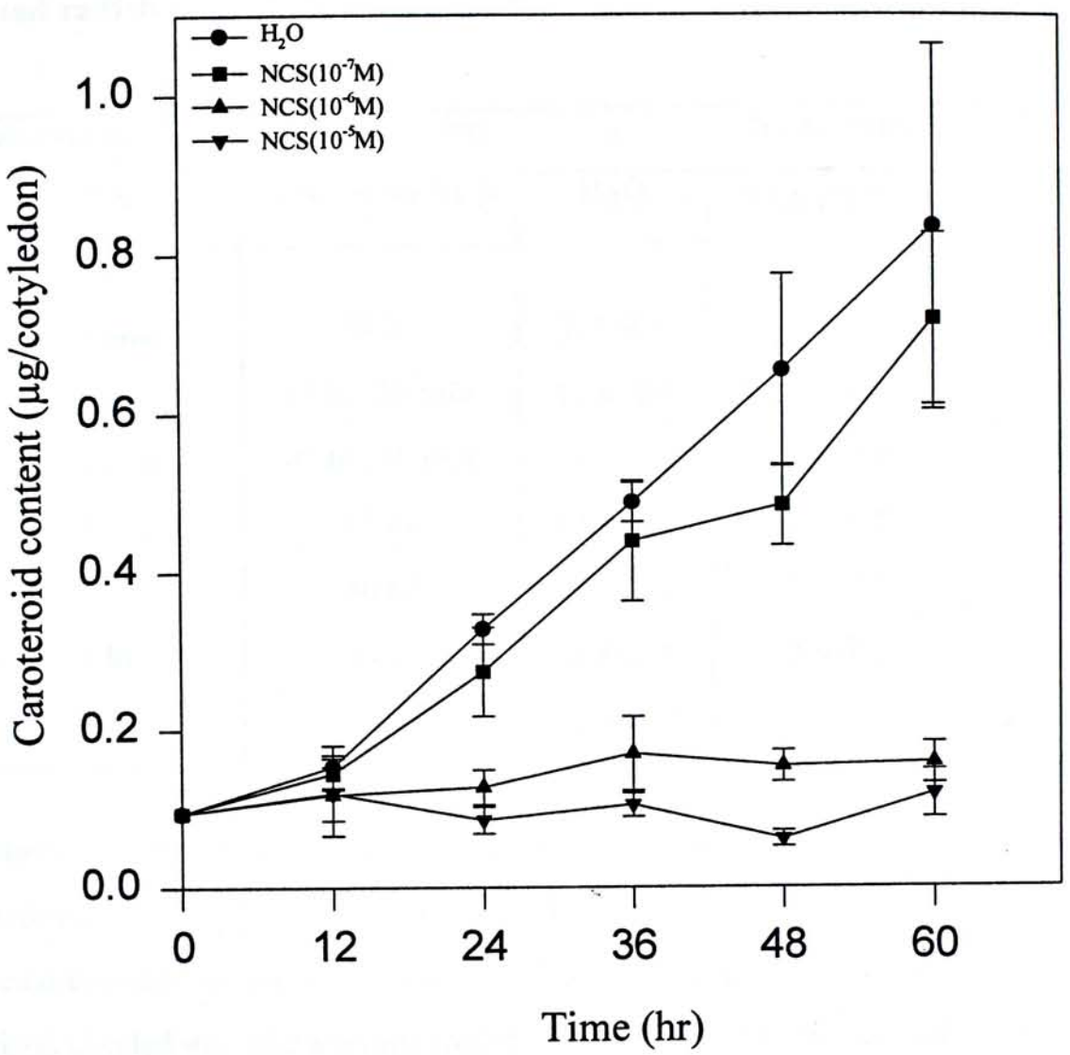


Fig. 4.18 Time course study of NCS on carotenoid content of excised radish cotyledons during growth in dark. Mean±SD, n=6.

Table 4.24 Time course study on the effect of BA pretreatment and NCS on excised radish cotyledons enlargement.

Pretreatment time in BA	Incubation time in water or NCS	Δ fresh weight (mg/cotyl.)		
		H_2O	NCS (10^{-6}M)	NCS (10^{-5}M)
BA (10^{-5}M);				
0 min	48 hr	8.7±0.9	1.3±0.4	0.8±0.2
5 min	47 hr, 55 min	10.4±0.5	1.4±0.1	0.5±0.1
30 min	47 hr, 30 min	11.9±1.3	1.4±0.2	0.7±0.1
1 hr	47 hr	12.7±1.2	1.6±0.2	0.6±0.1
2 hr	46 hr	13.6±3.9	2.3±0.2	0.7±0.2
4 hr	44 hr	14.9±2.1	3.9±0.1	1.7±0.1
48 hr	0	14.6±0.5		

Excised cotyledons were pretreated with 10^{-5} M BA for different times and then were transferred to distilled water, or 10^{-6}, 10^{-5}M NCS. The change of fresh weight of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. The average initial fresh weight of each excised cotyledon was 6.0-7.0mg and the change of fresh weight of excised cotyledons was expressed as mg/cotyl. Mean±SD, n=6.

Table 4.25 Time course study on the effect of BA pretreatment and NCS on the chlorophyll contents (μg/cotyledon) of excised radish cotyledons in light.

Pretreatment time in BA	Incubation time in water or NCS	Chlorophyll content (μg/cotyl.)		
		Chl a	Chl b	Total chl
BA => H_2O				
0 min	48 hr	11.18±1.96	4.98±0.85	16.31±2.83
5 min	47 hr, 55 min	10.67±0.33	4.50±0.27	15.32±0.60
30 min	47 hr, 30 min	11.04±1.28	4.40±1.23	15.61±0.63
1 hr	47 hr	11.44±0.41	4.53±0.79	16.14±1.21
2 hr	46 hr	11.88±0.41	5.42±0.22	17.49±0.62
4 hr	44 hr	12.86±0.90	5.66±0.79	18.72±1.70
48 hr	0 hr	15.09±3.25	5.59±0.73	20.82±4.02
BA =>NCS (10^{-6}M)				
0 min	48 hr	0.20±0.02	0.29±0.02	0.48±0.01
5 min	47 hr, 55 min	0.37±0.02	0.38±0.01	0.76±0.03
30 min	47 hr, 30 min	0.38±0.02	0.35±0.02	0.74±0.03
1 hr	47 hr	0.44±0.06	0.32±0.01	0.77±0.07
2 hr	46 hr	0.44±0.05	0.34±0.03	0.79±0.02
4 hr	44 hr	0.60±0.11	0.46±0.01	1.07±0.10
BA =>NCS (10^{-5}M)				
0 min	48 hr	0.07±0.02	0.06±0.01	0.13±0.03
5 min	47 hr, 55 min	0.10±0.0.01	0.16±0.01	0.26±0.02
30 min	47 hr, 30 min	0.09±0.02	0.11±0.02	0.20±0.30
1 hr	47 hr	0.13±0.04	0.22±0.06	0.36±0.10
2 hr	46 hr	0.24±0.04	0.40±0.07	0.64±0.11
4 hr	44 hr	0.30±0.01	0.48±0.01	0.79±0.02

Excised cotyledons were pretreated with 10^{-5}M BA for different times and then were transferred to distilled water, or 10^{-6}, 10^{-5}M NCS. Chlorophyll content of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. Mean±SD, n=6.

Table 4.26 presented the response of the growth of excised cotyledons in these experiments. The inhibitory effects of either 10^{-6} or 10^{-5}M NCS on the increase of fresh weight of excised cotyledons appeared from 5 min pretreatment. In 10^{-6}M NCS pretreated cotyledons for 5 min, inhibition of NCS to growth of cotyledons was about 23.2% comparison to that of control after growing in light for 48 hr, while in 10^{-5}M NCS pretreated cotyledons, the inhibition was approximately 25.5%. Cotyledons pretreated with 10^{-6}M NCS for different times were transferred to 10^{-5}M BA solution, the inhibition of NCS to expansion of excised radish cotyledons could be partially reversed. The expanding of cotyledons reached nearly the same fresh weights as those that were in control. Cotyledons pretreated with 10^{-5}M NCS showed the reversal of NCS-induced inhibition to expanding of cotyledons by BA was only efficient within 30 min pretreated with NCS, but the inhibitory effect of 10^{-5}M NCS producing after 1 hr could only be partially reversed and the growth rate was obviously decreased compared to control. When cotyledons were incubated in 10^{-5}M NCS for 4 hr in light at 28°C, these cotyledons growth were significantly inhibited, even if they were transferred to BA solution, the increase in fresh weight of those cotyledons was inhibited about 54.0% or 56.5% after being transferred to water or BA solution.

The similar effects were observed in chlorophyll formation during this period. As shown in Table 4.27, the greening of excised radish cotyledons in light was blocked by 10^{-6} and 10^{-5}M NCS pretreated for 30 min and longer. Inhibitions of NCS on chlorophyll formation increased with increasing concentrations of NCS and prolonging for

Table 4.26 Time course study on the effects of NCS pretreatment on growth of excised radish cotyledons.

Pretreatment time in NCS		Incubation time in water or BA	Δ fresh weight (mg /cotyl.)	
			H_2O (Ctrl)	BA (10^{-5}M)
10^{-6}M;	0 min	48 hr	10.40±0.76	15.00±0.99
	5 min	47 hr, 55 min	7.99±0.38	14.72±0.46
	30 min	47 hr, 30 min	7.82±0.52	13.98±0.35
	1 hr	47 hr	7.49±0.08	12.91±0.36
	2 hr	46 hr	7.42±0.36	12.65±0.87
	4 hr	44 hr	7.33±0.61	12.66±0.21
	48 hr	0	2.86±0.25	
10^{-5}M;				
	5 min	47 hr, 55 min	7.75±0.25	14.56±0.32
	30 min	47 hr, 30 min	7.56±0.74	12.55±0.32
	1 hr	47 hr	4.82±0.28	9.40±0.79
	2 hr	46 hr	4.63±0.81	7.63±0.33
	4 hr	44 hr	4.78±0.49	6.52±0.17
	48 hr	0	1.28±0.16	

Excised cotyledons were pretreated with 10^{-6} or 10^{-5}M NCS for different times and then transferred to distilled water, or 10^{-5}M BA. The change of fresh weight of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. The average initial fresh weight of each excised cotyledon was 6.0-7.0mg. The change of fresh weight of excised cotyledons was expressed as mg/cotyl. Mean±SD, n=6.

Table 4.27 Time course study on the effects of pretreatment with NCS on chlorophyll contents (μg /cotyl.) of excised radish cotyledons.

Pretreatment time in NCS	Incubation time in water or BA	Chlorophyll content in H_2O (μg/cotyl.)			Chlorophyll content in BA (μg/cotyl.)		
		Chl a	Chl b	Total chl	Chl a	Chl b	Total chl
NCS (10^{-6}M)							
=> 0 min	48 hr	12.52±1.95	5.59±085	18.31±2.83	15.09±3.25	6.72±0.73	21.93±2.69
5 min	47 hr, 55 min	12.64±2.40	5.35±0.87	18.18±3.30	14.47±1.00	5.32±1.43	19.98±2.45
30 min	47 hr, 30 min	12.24±0.49	3.98±0.96	16.40±126	12.86±0.57	5.49±0.59	18.53±1.17
1 hr	47 hr	11.19±0.37	4.01±0.30	15.25±0.51	12.17±1.21	5.01±0.30	17.52±1.02
2 hr	46 hr	10.17±2.11	4.10±0.70	14.42±2.90	11.96±2.04	4.13±1.18	16.27±2.26
4 hr	44 hr	10.11±2.10	3.90±1.37	14.17±2.34	11.60±2.03	4.88±1.15	16.65±2.41
48 hr	0	0.40±0.10	0.27±0.10	0.68±0.2			
NCS (10^{-5}M)							
=> 5 min	47 hr, 55 min	9.69±0.44	4.23±.031	14.07±0.76	12.64±0.53	5.30±0.33	18.13±0.64
30 min	47 hr, 30 min	4.91±0.22	2.03±0.32	7.03±0.67	5.81±0.41	2.37±0.50	8.27±0.85
1 hr	47 hr	2.71±0.20	1.32±0.74	4.08±0.45	2.77±0.35	1.36±0.36	4.16±0.72
2 hr	46 hr	1.69±0.26	0.87±0.18	2.59±0.44	1.98±0.28	0.80±0.27	2.81±0.56
4 hr	44 hr	1.81+0.60	0.91±0.35	2.74±0.66	1.87±0.03	0.91±0.05	2.81±0.08
48 hr	0	0.08±0.03	0.07±0.02	0.15±0.05			

Excised cotyledons were pretreated with 10^{-5}M or 10^{-6}M NCS for different times and then were transferred to distilled water, or 10^{-5}M BA. Chlorophyll content of excised cotyledons was determined after incubation for 48 hr in light. Each sample included ten excised cotyledons. Mean±SD, n=6.

pretreatment times. The inhibition of 10^{-6}M and 10^{-5}M NCS on total chlorophyll contents after pretreatment for 4 hr was around 22.6% and 85.0% contrast to control when they were transferred to water. There were about 9.1% and 84.7% inhibitions on total chlorophyll contents comparing to control, when they were transferred to BA solution incubation.

VI. Interaction between NCS and phytohormones in growth and greening of excised radish cotyledons

A. Effects of BA, GA_3 and ABA on the growth and greening of excised radish cotyledons

Table 4.28 presented the effects of different concentrations of BA, GA_3 and ABA on the growth of excised radish cotyledons in light for 48 hr at 28°C. In these experiments, 10^{-5}M BA and 10^{-6}M GA_3 showed the maximum stimulation on the cotyledons growth, approximately 218.9% and 147.9%. ABA (10^{-6}M and 10^{-5}M) inhibited by 23.9% and 57.0% in cotyledons growth incubated for 48 hr in light at 28°C. The similar results were observed in greening of excised radish cotyledons (Table 4.29).

The inhibitory effect of ABA on either expansion or greening of excised radish cotyledons was partially reversed by BA or GA_3. For expansion of cotyledons, the

Table 4.28 Effect of different concentrations of BA, GA_3 and ABA on growth of excised radish cotyledons under light for 48 hr

Concentration (M)	Δ fresh weight (mg/cotyl.)		
	BA	GA_3	ABA
0	8.72±0.62	8.72±0.62	8.72±0.62
10^{-7}	11.88±2.25	11.28±0.17	8.64±0.99
10^{-6}	14.63±1.93	12.90±0.74	6.29±0.82
10^{-5}	19.09±2.92	11.00±0.64	3.75±0.83

Excised radish cotyledons were obtained from radish seeding after for 48 hr germination in darkness at 22±2°C. Excised cotyledons were incubated in light (50uE-2s-1, PAR) in various concentration of BA, GA_3 or ABA, or both BA and ABA, GA_3 and ABA, for 48 hr at 28°C. Change of fresh weight of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. The average initial fresh weight of each excised cotyledon was 6.0-7.0mg. Mean±SD, n=6.

Table 4.29 Effects of different concentrations of BA, GA_3 and ABA on chlorophyll contents (μg/cotyl.) of excised radish cotyledons for 48 hr in light.

Concentration (M)		Chlorophyll content (μg/cotyl.)		
		Chl a	Chl b	Total chl
0		14.04±1.29	4.35±0.38	18.60±1.69
BA;	10^{-7} (M)	14.55±1.40	4.45±0.37	19.22±1.78
	10^{-6} (M)	15.38±0.98	4.42±0.27	20.03±1.23
	10^{-5} (M)	17.38±2.64	4.93±0.62	22.57±0.26
GA_3;	10^{-7} (M)	15.89±0.64	4.99±0.15	21.12±0.80
	10^{-6} (M)	16.83±0.19	5.11±0.16	22.18±0.35
	10^{-5} (M)	15.15±0.12	5.12±0.07	20.42±0.06
ABA;	10^{-7} (M)	14.41±3.28	4.94±0.66	19.57±3.83
	10^{-6} (M)	12.22±2.01	4.51±0.84	16.92±2.52
	10^{-5} (M)	8.23±1.88	2.87±0.45	11.22±2.26

Excised radish cotyledons were obtained from radish seeding after 48 hr germination in darkness at 22±2°C. Excised cotyledons were incubated in light (50uE-2s-1, PAR) in various concentration of BA, GA_3 or ABA for 48 hr at 28°C. Chlorophyll content of excised cotyledons was determined after incubation for 48 hr. Control was incubated in distilled water. Each sample included ten excised cotyledons. Mean±SD, n=6.

simultaneous addition of 10^{-5}M BA could reverse the inhibition caused by 10^{-6}M ABA (Table 4.30). Inhibition of 10^{-5}M ABA on cotyledon expansion could partially be reversed by BA, (from 45.7% inhibition to 12.7%). Compared to BA, GA_3 was less effective in reversing the inhibitory effect caused by ABA. As shown in Table 4.31, inhibition of ABA (10^{-6}M and 10^{-5}M) on greening of excised radish cotyledons could also be reversed by 10^{-5} M BA, partially. GA_3 showed less effective in reversing the inhibitory effect caused by ABA than that of BA.

B. Interaction of NCS with phytohormones on growth and greening of excised cotyledons

Table 4.32 and Table 4.33 showed the effects of NCS, BA, GA_3 and ABA on expansion and greening of excised radish cotyledons after incubation for 48 hr in light at 28°C. Inhibitory effects of NCS (10^{-6}M and 10^{-5}M) either in expansion of excised cotyledons could hardly be reversed by BA (10^{-5}M) or GA_3 (10^{-6}M). ABA seemed less effective either in cotyledon expansion or in greening. On the other hand, 10^{-6}M ABA could not enhance the inhibitory effect of NCS (10^{-6}M and 10^{-5}M) either in cotyledon expansion or in greening after incubation for 48 hr in light at 28°C. The inhibition caused by 10^{-6}M NCS on chlorophyll content could be reversed only a little by BA or GA_3. The total chlorophyll content was increased by BA or GA_3, from 0.46 to 1.39μg/cotyl or from 0.46 to 0.81 μg/cotyl., respectively.

Table 4.30 Effects of the interactions of ABA and BA , GA_3 on the growth of excised radish cotyledons after 48 hr in the light.

Treatment	Δ fresh weight (mg/cotyl.)		
	0	BA (10^{-5} M)	GA_3 (10^{-6} M)
0	9.37±0.56	17.85±0.51	12.04±1.87
ABA (10^{-6} M)	6.71±0.45	9.93±0.53	8.64±0.60
ABA (10^{-5} M)	5.09±0.17	8.18±0.19	5.91±0.25

Excised radish cotyledons were obtained from radish seeding after 48 hr germination in darkness at 22±2°C. Excised cotyledons were incubated in light (50uE-2s-1, PAR) in various concentration of BA, GA_3 or ABA, or both BA and ABA, GA_3 and ABA for 48 hr at 28°C. Change of fresh weight of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. The average initial fresh weight of each excised cotyledon was 6.0-7.0mg. Mean±SD, n=6.

Table 4.31 Effects of the interactions of ABA and BA , GA_3 on the chlorophyll contents (μg/cotyl.) of excised radish cotyledons after 48 hr in light.

Treatment	Chlorophyll content (μg/cotyl.)		
	Chl a	Chl b	Total chl
0	15.30±2.64	4.59±0.80	20.12±3.48
BA (10^{-5} M)	17.78±1.84	5.24±0.45	23.28±2.14
GA_3 (10^{-6} M)	15.45±2.06	4.84±0.66	20.52±2.75
ABA (10^{-6} M)	13.17±3.34	4.20±0.42	17.57±4.49
ABA (10^{-5} M)	10.14±1.77	3.24±0.60	13.53±2.40
BA (10^{-5} M) +			
ABA (10^{-6} M)	14.24±1.24	4.34±0.42	18.79±1.65
ABA (10^{-5} M)	12.28±1.04	3.75±0.31	16.21±1.36
GA_3 (10^{-6} M) +			
ABA (10^{-6} M)	13.59±0.14	4.30±0.05	18.08±0.14
ABA (10^{-5} M)	10.28±1.14	3.27±0.44	13.71±1.60

Excised radish cotyledons were obtained from radish seeding after 48 hr germination in darkness at 22±2°C. Excised cotyledons were incubated in light (50uE-2s-1, PAR) in various concentration of BA, GA_3 or ABA, or both BA and ABA, GA_3 and ABA for 48 hr at 28°C. Chlorophyll content of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. Mean±SD, n=6.

Table 4.32 Effects of NCS, ABA, BA and GA_3 on the increased fresh weight (mg/cotyl.) of excised radish cotyledons for 48 hr in light.

Treatment	Δ fresh weight (mg/cotyl.)		
	NCS (M)		
	0	10^{-6}	10^{-5}
0	8.54±0.43	1.95±0.24	0.74±0.11
BA (10^{-5} M)	15.07±0.49	2.30±0.40	0.91±0.18
GA_3 (10^{-6} M)	10.84±0.77	2.87±0.54	0.78±0.18
ABA (10^{-6}M)	6.71±0.45	1.82±0.30	0.72±0.18

Excised radish cotyledons were obtained from radish seeding after 48 hr germination in darkness at 22±2°C. Excised cotyledons were incubated in light (50uE-2s-1, PAR) in various concentration of NCS, BA, GA_3 or ABA, or both NCS and BA, NCS and GA_3, NCS and ABA for 48 hr at 28°C. Change of fresh weight of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. The average initial fresh weight of each excised cotyledon was 6.0-7.0mg. Mean±SD, n=6.

Table 4.33 Effects of the interactions of NCS, BA , GA_3 and ABA on the chlorophyll contents

Treatment	Chlorophyll content (μg/cotyl.)		
	Chl a	Chl b	Total chl
0	12.55±0.82	3.88±0.18	16.62±1.01
BA (10^{-5}M)	17.85±0.03	4.87±0.06	22.50±0.03
GA_3 (10^{-6}M)	15.85±1.77	4.83±0.60	20.92±2.40
ABA (10^{-6}M)	9.85±0.63	3.12±0.08	13.12±0.69
NCS (10^{-6}M)	0.32±0.16	0.14±0.03	0.46±0.18
NCS (10^{-5}M)	0.07±0.01	0.09±0.03	0.16±0.03
BA (10^{-5} M) + NCS (10^{-6}M)	1.05±0.07	0.33±0.04	1.39±0.11
NCS (10^{-5}M)	0.11±0.05	0.10±0.45	0.22±0.07
GA_3 (10^{-6} M) + NCS (10^{-6}M)	0.58±0.28	0.22±0.09	0.81±0.37
NCS (10^{-5}M)	0.09±0.03	0.10±0.08	0.19±0.09
ABA (10^{-6}M) + NCS (10^{-6}M)	0.33±0.15	0.13±0.08	0.48±0.23
NCS (10^{-5}M)	0.06±0.02	0.10±0.04	0.16±0.06

Excised radish cotyledons were obtained from radish seeding after 48 hr germination in darkness at 22±2°C. Excised cotyledons were incubated in light (50uE-2s-1, PAR) in various concentration of NCS, BA, GA_3 or ABA, or both NCS and BA, NCS and GA_3, NCS and ABA for 48 hr at 28°C. Chlorophyll content of excised cotyledons was determined after incubation for 48 hr. Each sample included ten excised cotyledons. Mean±SD, n=6.

4.3. Investigation of effects of NCS on chlorophyll synthesis

I. Effect of preincubation in water on growth and greening of excised cotyledons under light

Fig. 4.19 showed that preincubation in water for 12 hr and 24 hr reduced NCS-induced inhibitory effect on growth of excised radish cotyledons, especially in the 24 hr preincubation treatment. Inhibition caused by 10^{-6}M NCS on growth of excised radish cotyledons was decreased from 78.6% (without preincubated) to 39.1% (preincubation in water) for 12 hr after they were transferred to 10^{-6}M NCS for 24 hr. Inhibition on growth of excised radish cotyledons caused by 10^{-5}M NCS was reduced from 91.8% to 53.3%. Cotyledons preincubated in distilled water for 24 hr were compared to the cotyledon incubated with NCS (10^{-6}M or 10^{-5}M) directly. The inhibition on growth of cotyledons caused by 10^{-6} M NCS decreased from 80.2% to 5.9% after they were transferred to 10^{-6}M NCS for 24 hr, the inhibition caused by 10^{-5}M NCS decreased from 92.1% to 19.2%. For the greening of excised cotyledons (Fig. 4.20), only preincubation in water for 24 hr reduced the inhibitions caused by 10^{-6} and 10^{-5}M NCS on chlorophyll content after they were transferred to NCS solutions for 12 hr, but in prolonging incubation with NCS for 24 hr, the chlorophyll contents of cotyledons were decreased.

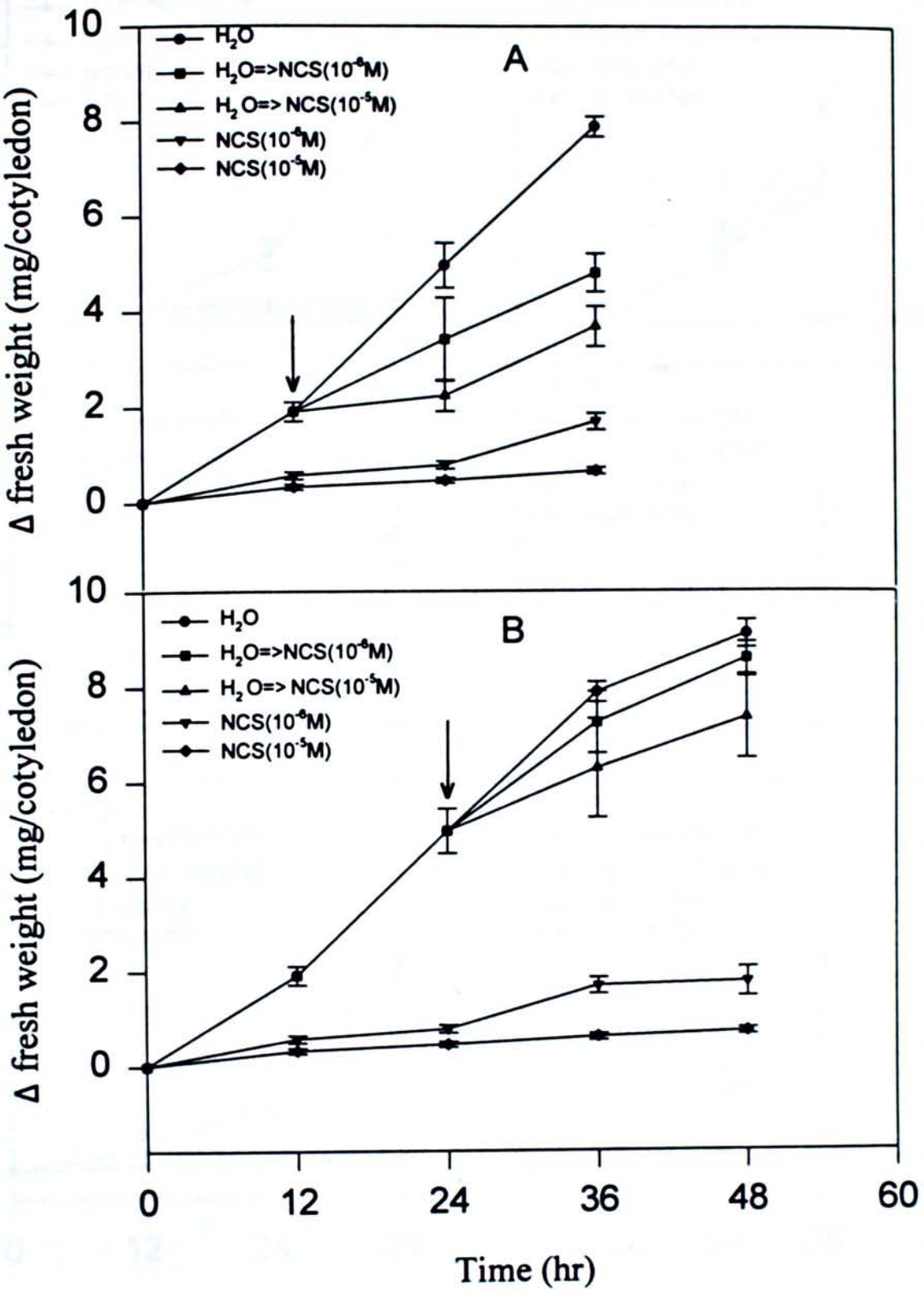


Fig. 4.19 Effect of NCS on the growth of excised radish cotyledons after preincubation in water for different times. Excised cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C. The average initial fresh weight of excised cotyledon was 6.0-7.0mg/cotyledon. Arrow indicates the time of transfer from water to NCS. Mean±SD, n=6. Pretreatment with water for 12 hr; A,C,E; for 24 hr; B,D,F.

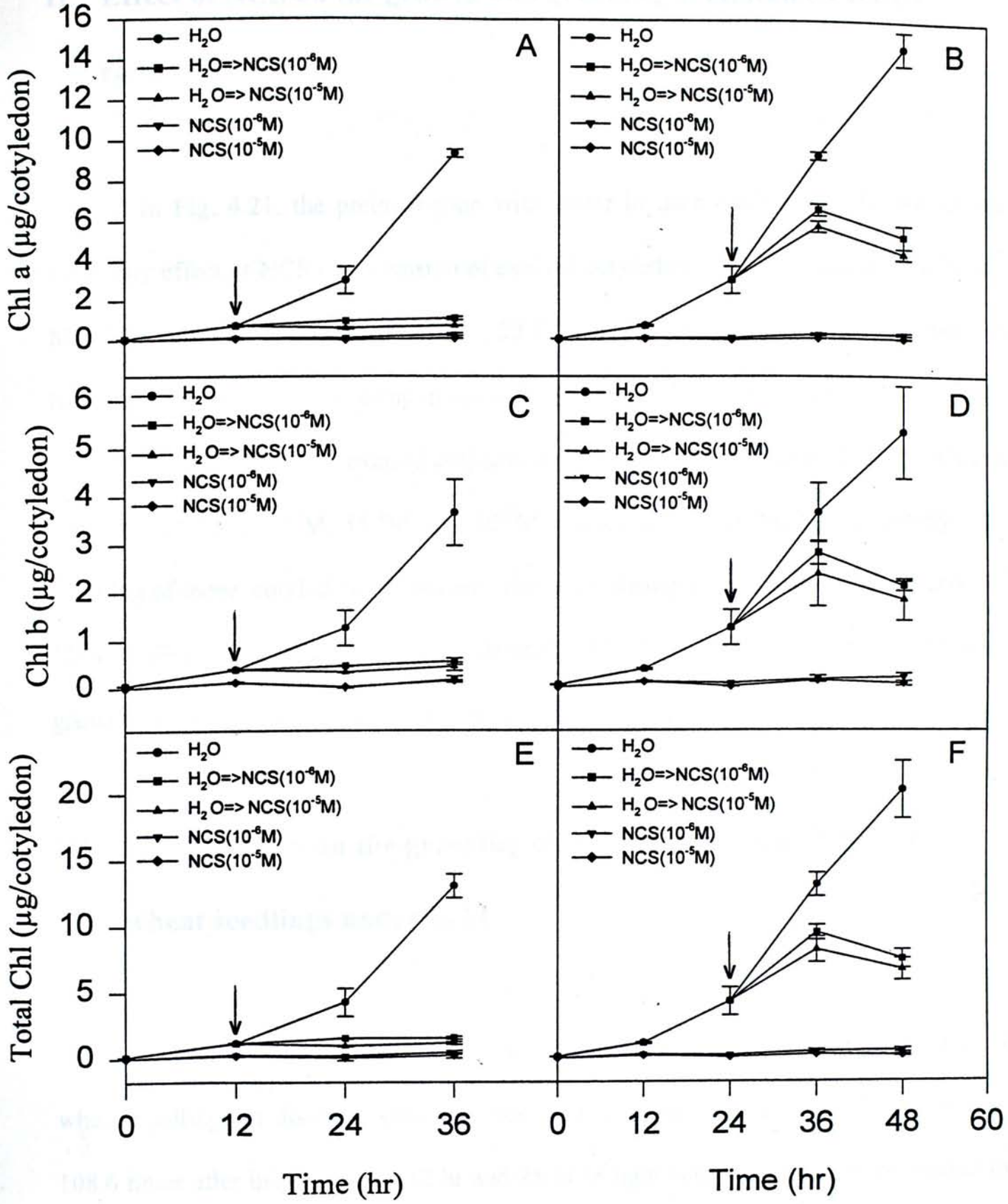


Fig. 4.20 Effect of NCS on the chlorophyll content of excised radish cotyledons after preincubation in water for different times. Excised cotyledons were obtained from radish seeds after germination in dark at 22+2°C. Mean±SD, n=6.

Arrow indicates the time of transfer from water to NCS.
Pretreatment with water for 12 hr; A,C,E; for 24 hr; B,D,F.

II. Effect of NCS on the growth and greening of etiolated excised radish cotyledons

In Fig. 4.21, the preincubation with water in dark could markedly reduce the inhibitory effect of NCS on expansion of excised cotyledons. The inhibitions of NCS 10^{-7} M, 10^{-6}M and 10^{-5}M were about 11.0%, 20.1% and 34.7% when they were incubated in light for 12 hr after preincubating in water for 48 hr in dark. But there were about 39.3%, 53.3% and 56.6% in those excised cotyledons without preincubating in dark, growing in light for 12 hr at 10^{-7}M, 10^{-6}M and 10^{-5}M concentrations of NCS, respectively. The greening of those cotyledons, however, was still strongly inhibited by 10^{-6}M and 10^{-5} M NCS (Fig. 4.22). There were approximately 25%, 84% and 95% in those cotyledons growing for 12 hr after preincubation 48 hr in dark, respectively.

III. Effect of NCS on the greening of etiolated leaves of 7-day-old wheat seedlings under light

On continuous illumination, the total chlorophyll content of etiolated leaves of wheat seedlings in distilled water (control) increased rapidly, reaching 28.8 times and 108.6 times after incubation for 12 hr and 48 hr in light (Fig. 4.23). Inhibition caused by NCS to chlorophyll content increased with increasing concentrations. The total chlorophyll contents reached 21.9, 2.6 and 0.9 times for 12 hr in light at 10^{-7}, 10^{-6} and

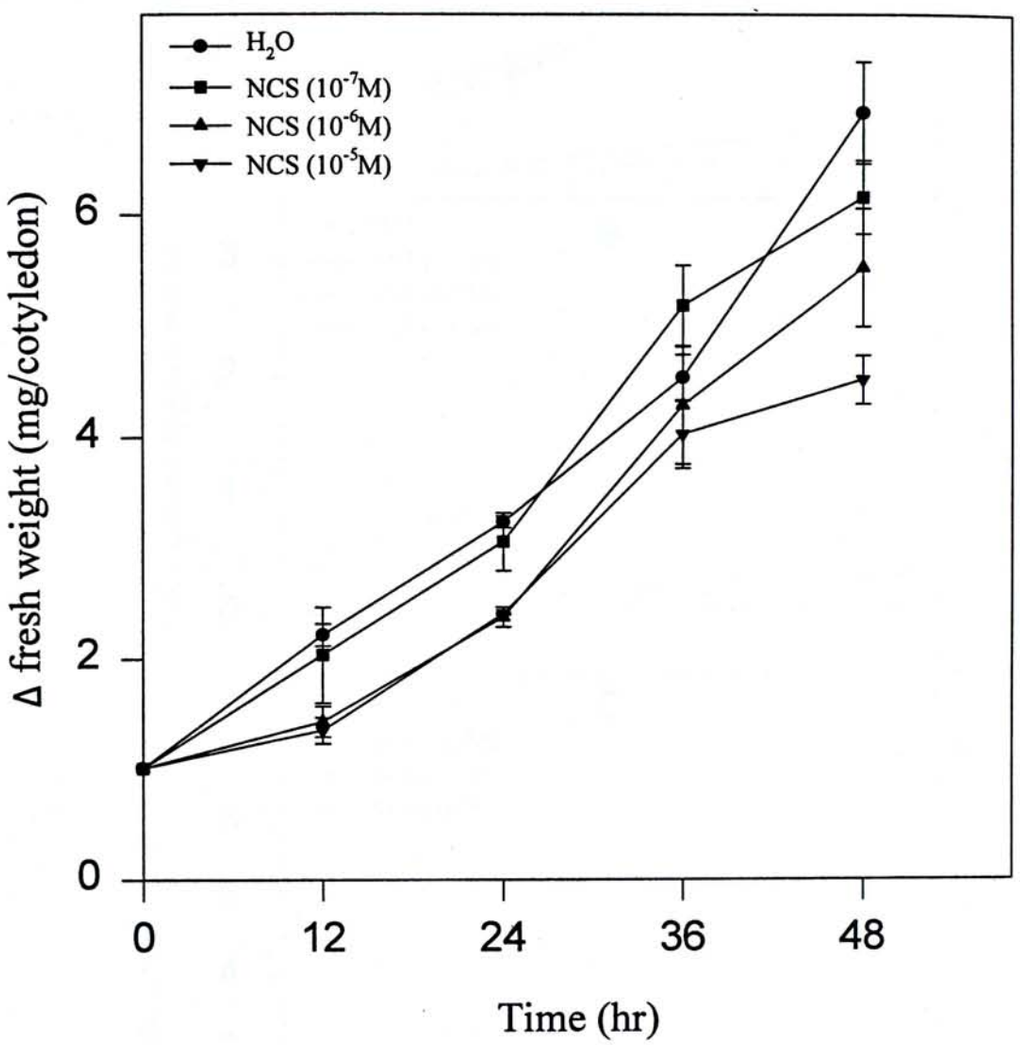


Fig. 4.21 Effect of NCS on growth of excised radish cotyledons under light for 12 hr after preincubation in water for different times in dark. The average initial fresh weight of excised cotyledon was 6.0-7.0mg/cotyledon. Mean±SD, n=6.

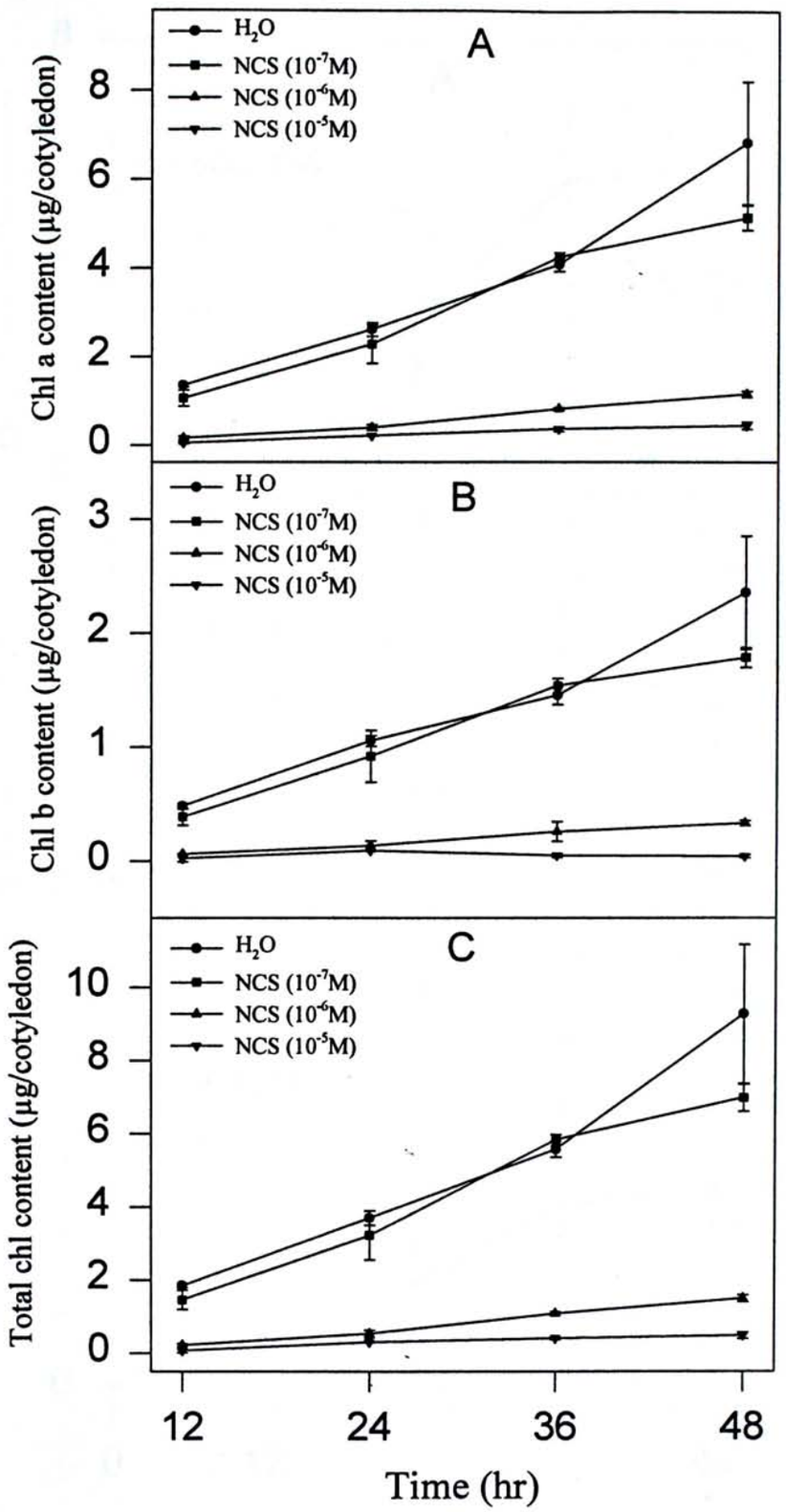


Fig. 4.22 Effect of NCS on chlorophyll contents of excised radish cotyledon after preincubation in water for different times in dark, before transferring to light for 12 hr.
A: chl a; B: chl b; C: total chl. Mean±SD, n=6.

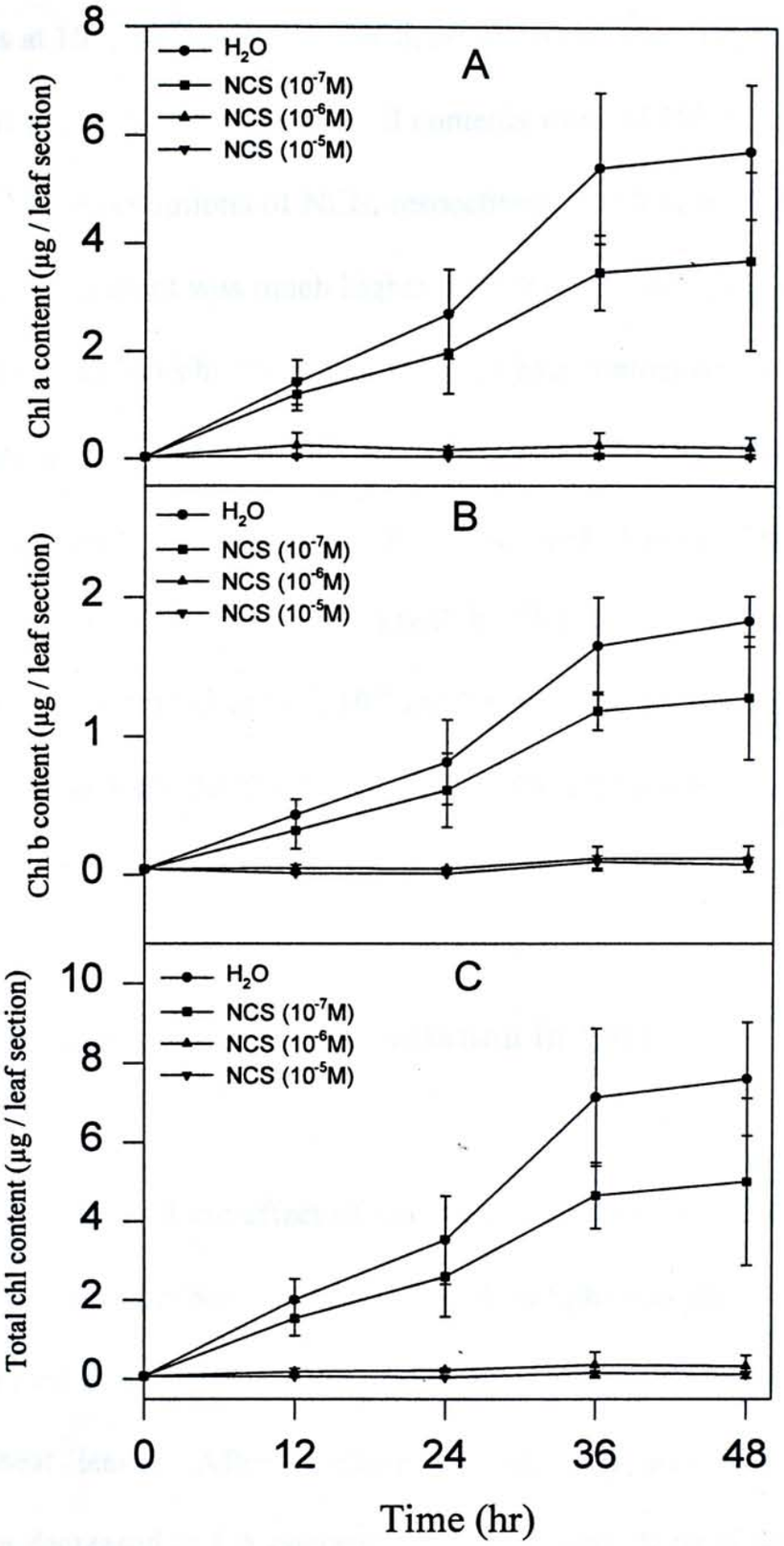


Fig. 4.23 Time course study of NCS on chlorophyll content of etiolated leaves of wheat growing under light. Etiolated leaves were obtained from 7-day-old wheat seedlings germinated in dark. Mean±SD, n=6.
A: chl a; B: chl b; C: total chl.

10^{-5} M NCS, respectively. However, they were only increased approximately 71.5, 4.6 and 1.6 times at 10^{-7}, 10^{-6} and 10^{-5}M NCS, respectively, after incubation for 48 hr in light. Inhibition caused by NCS to chlorophyll contents were 34.1%, 95.8% and 98.5% at 10^{-7}, 10^{-6} and 10^{-5}M concentrations of NCS, respectively, at 48 hr in light. On the other hand, increase of Chl a content was much higher than that of Chl b after the leaves of etiolated wheat were exposed to light. In control leaves, Chl a content reached a value 189.7 times after exposure to light for 48 hr, but Chl b content only reached 10.8 times. NCS (10^{-6} and 10^{-5}M) completely blocked both Chl a and Chl b. Chlorophyll a exhibited more sensitivity to NCS than chlorophyll b. There were 35.5%, 96.4% and 99.3% inhibitions to Chl a contents at 10^{-7}, 10^{-6} and 10^{-5}M NCS after exposure to light for 48 hr, respectively. There were 30.2% 93.8% and 96.0% inhibitions to Chl b contents at 10^{-7}, 10^{-6} and 10^{-5}M NCS after exposure to ligth for 48 hr, respectively.

IV. Effect of LA on ALA accumulation in light

Fig. 4.24 showed the effect of various concentrations of LA on the formation of ALA and Chl. Maximum accumulation of ALA in light was observed at 25mM of LA in this study. From 0 to 25mM of LA, there was an increase in ALA accumulation in etiolated wheat leaves. After reaching a maximum accumulation of ALA, ALA accumulation decreased as LA concentrations increased. A rapid decrease in chlorophyll content corresponded to the increase of ALA up to 25mM LA.

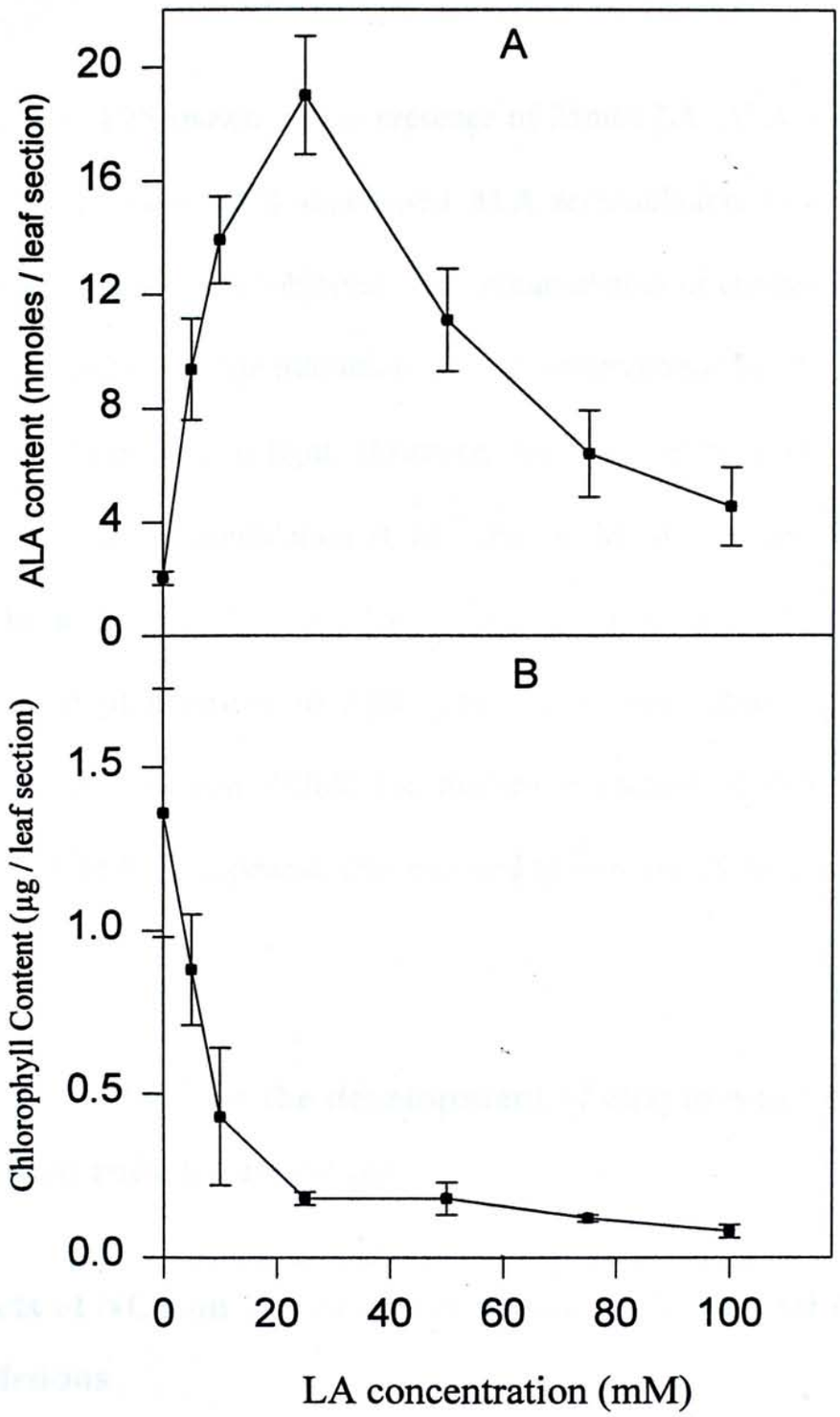


Fig. 4.24 Effect of LA on ALA accumulation (A) and chlorophyll content (B) of etiolated leaves of wheat. Seven-day-old etiolated leaves were preincubated in dark for 3 hr in increasing concentrations of LA and were then illuminated for 8 hr. Mean±SD, n=6.

V. Time course study of NCS on ALA accumulation in the presence of LA

As Fig. 4.25 shown, in the presence of 25mM LA, ALA accumulation increased with time in the light. NCS suppressed ALA accumulation, especially at 10^{-6}M NCS. NCS (10^{-6}M) significantly inhibited ALA accumulation of etiolated wheat leaves during the period of treatment. The inhibition of ALA accumulation by 10^{-6} M NCS appeared in early stage of exposure to light. However, there was approximately 23.2% and 76.4% inhibitions of ALA accumulation at 10^{-7} and 10^{-6}M NCS respectively after exposed to light for 16 hr. The inhibition of chlorophyll accumulation by 10^{-6}M NCS also appeared in early stage of exposure to light. There was approximately 44.1% inhibition on chlorophyll accumulation. While the marked inhibition of chlorophyll accumulation caused by 10^{-7}M NCS appeared after exposed to light for 24 hr, the inhibition was about 42.1%.

4.4 Effect of NCS on the development of enzymes activities in the excised radish cotyledons

I. Effects of NCS on isocitrate lyase activity of excised radish cotyledons

Effect of NCS on isocitrate lyase activity was shown in Fig. 4.26. In control, the activity of isocitrate lyase reached a maximum at 24 hr, then decreased. NCS repressed

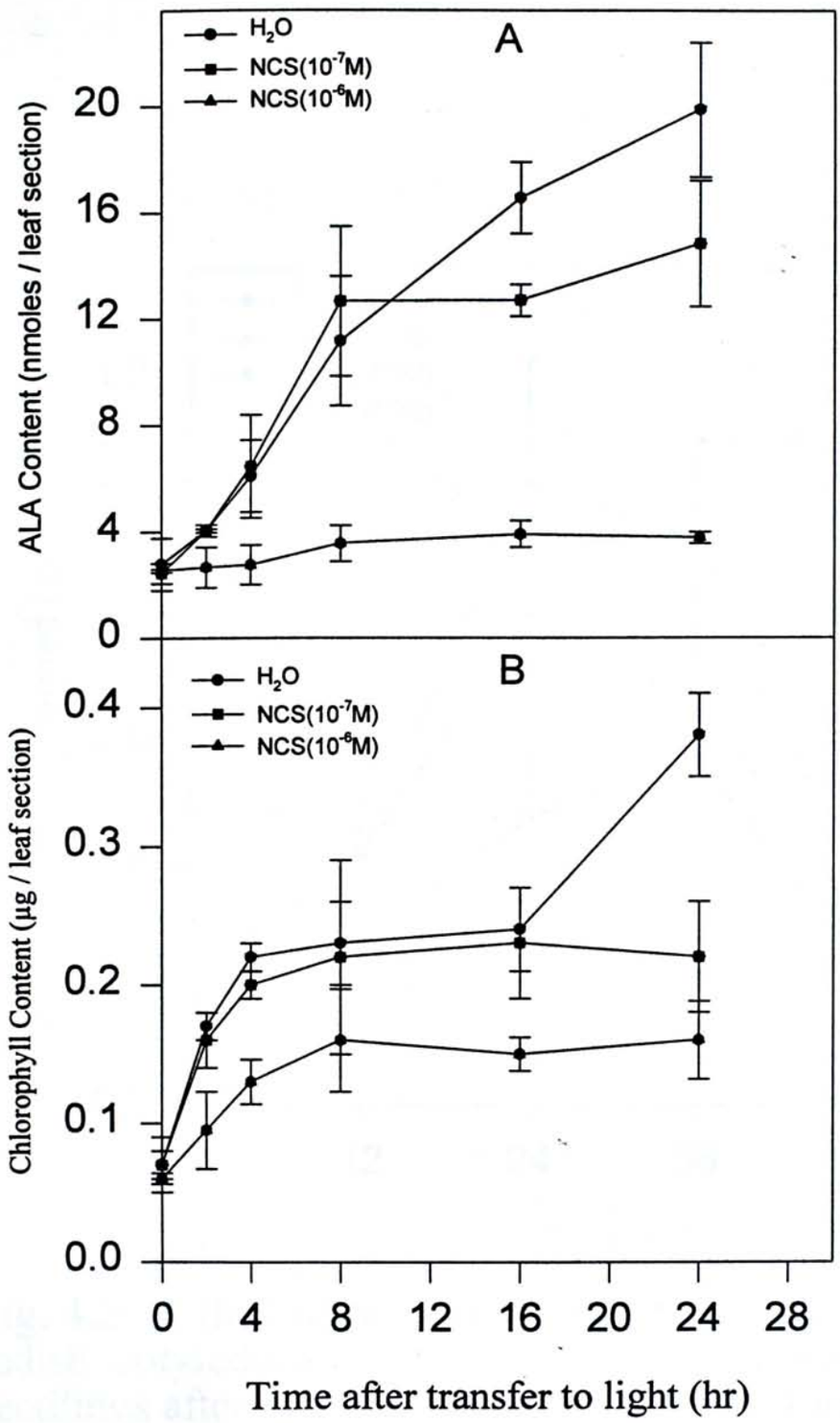


Fig. 4.25 Effects of LA and NCS on accumulation of ALA and Chl in greening of etiolated wheat leaves. Seven-day-old etiolated leaves were preincubated in 25mM LA and various concentrations of NCS at pH5.4 for 3 hr in dark and then were transferred to light for different times. Mean±SD, n=6.

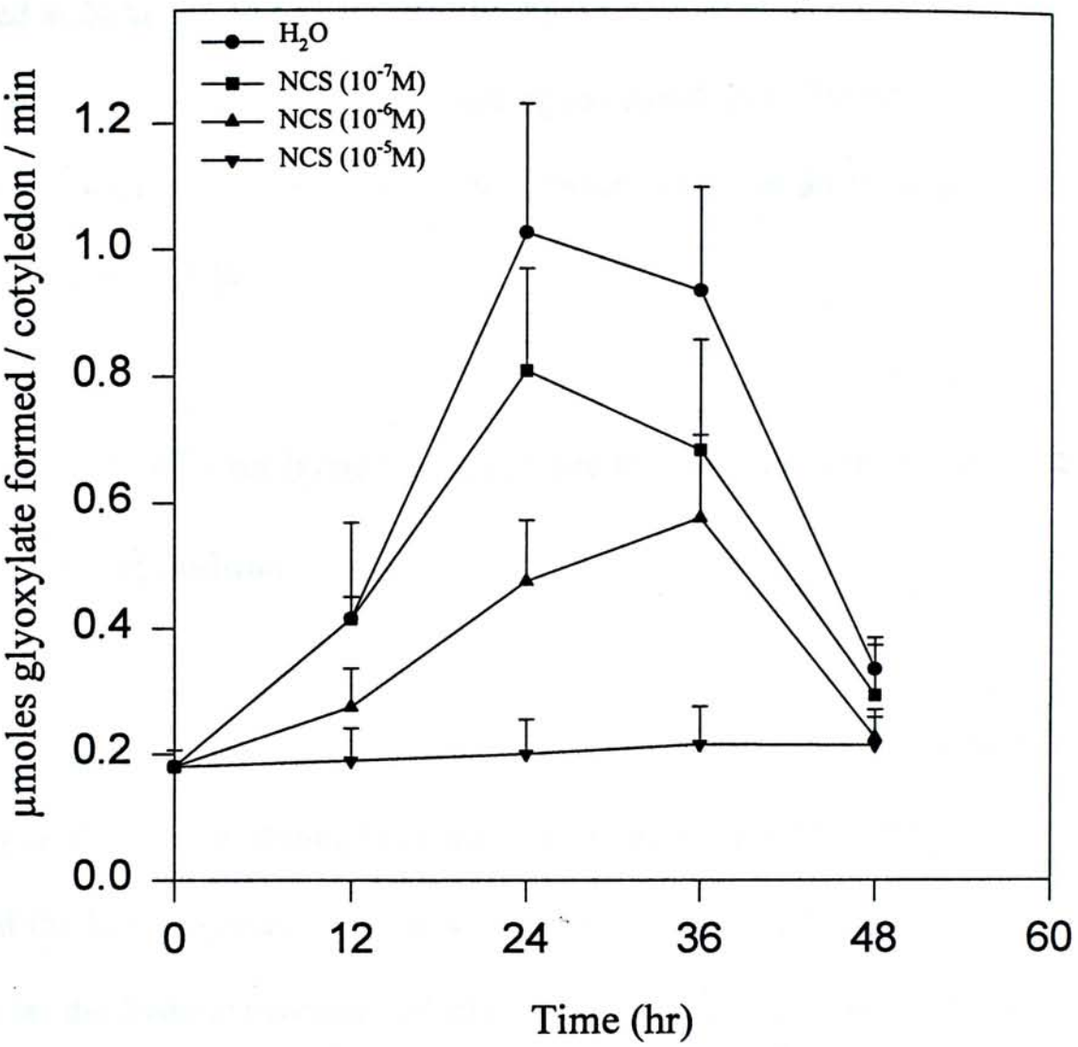


Fig. 4.26 Effect of NCS on isocitrate lyase activity in excised radish cotyledons. Excised cotyledons were obtained from seedlings after 48 hr germination. Mean±SD, n=6.

isocitrate lyase activity. The inhibition of isocitrate lyase activity by NCS increased with increasing concentrations. NCS (10^{-5}M) completely inhibited isocitrate lyase activity. Cotyledons treated with 10^{-7} and 10^{-6}M NCS, a peak of isocitrate lyase activity was observed at 24 hr and 36 hr, respectively. The inhibition of 10^{-7}M NCS to isocitrate lyase activity at 24 hr was about 21.3% comparing to control, in 10^{-6}M NCS treated cotyledons, the inhibition of this enzyme activity was about 44.0% at 36 hr in comparison to the peak of control at 24 hr.

II. Effect of NCS on hydroxypyruvate reductase activity of excised radish cotyledons

In contrast to isocitrate lyase, the activity of hydroxypyruvate reductase increased steadily in the control during incubation up to 48 hr in light. NCS (10^{-5}M) completely blocked the hydroxypyruvate reductase activity. Inhibitions of approximately 4.9% and 77.3% on the hydroxypyruvate reductase activity were observed in cotyledons treated with 10^{-7} and 10^{-6}M NCS after 48 hr incubation in light, respectively (Fig. 4.27 and Fig. 4.28).

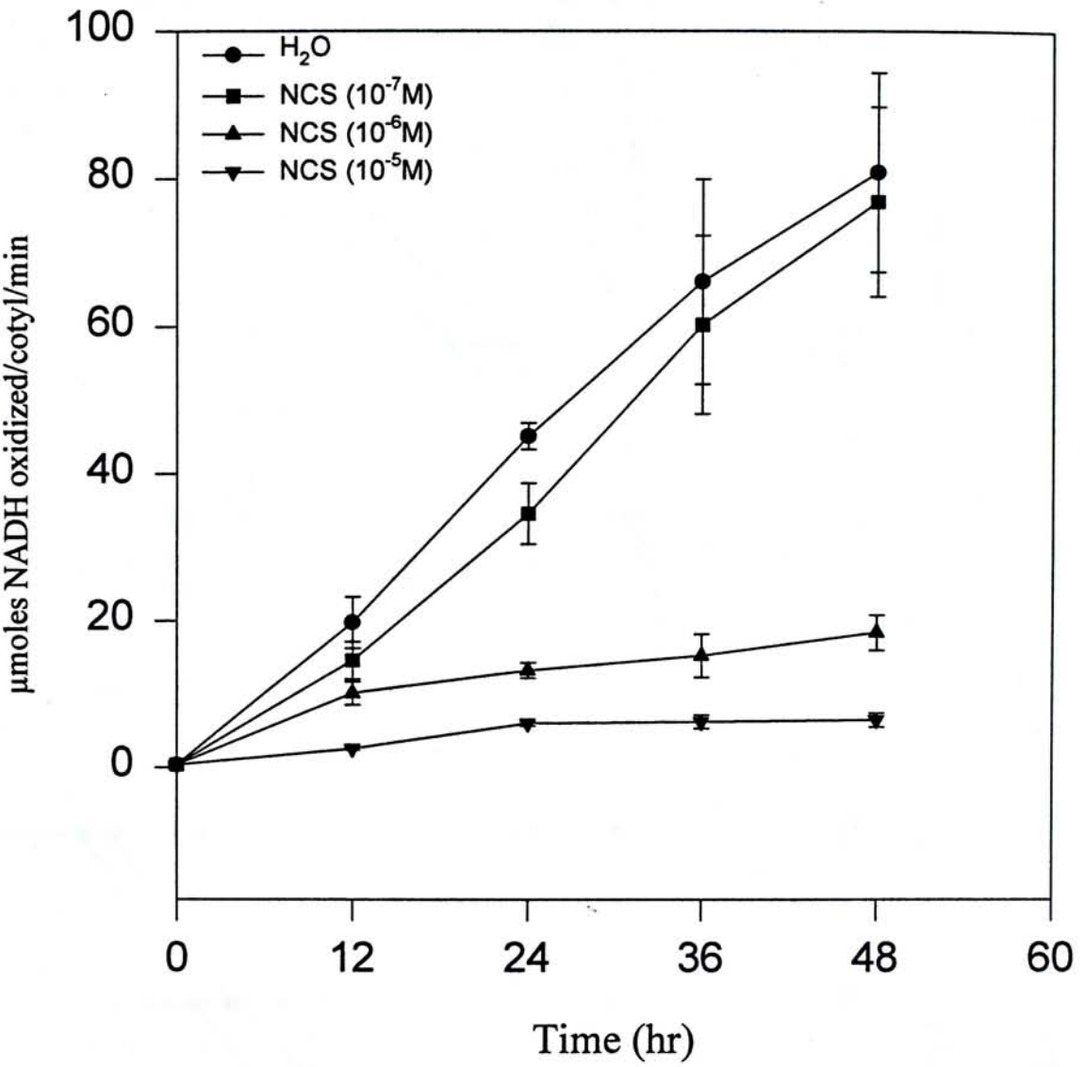


Fig. 4.27 Effect of NCS on hydroxypyruvate reductase activity in radish cotyledons. Excised radish cotyledons were obtained after 48 hr germination. Mean±SD, n=6.

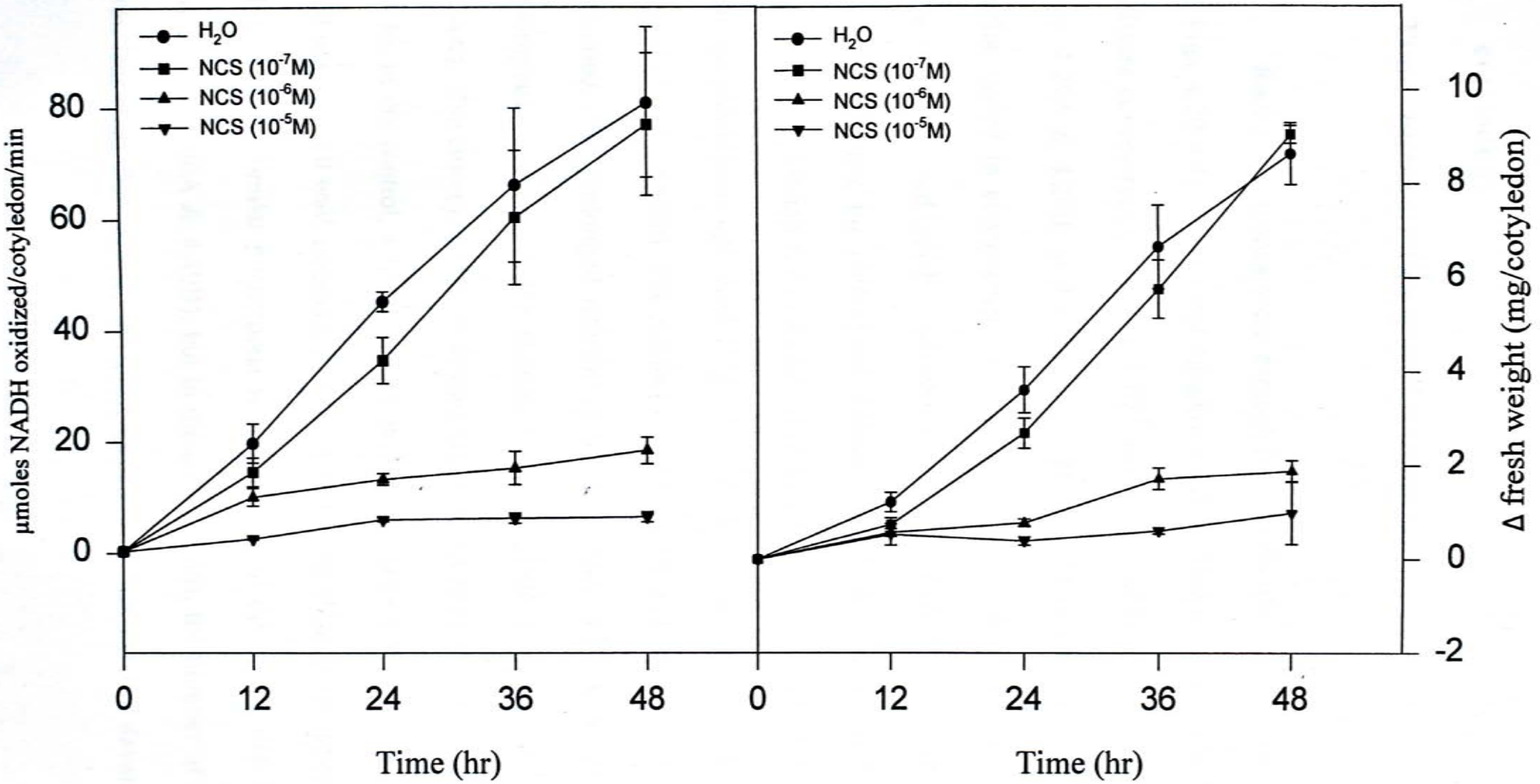
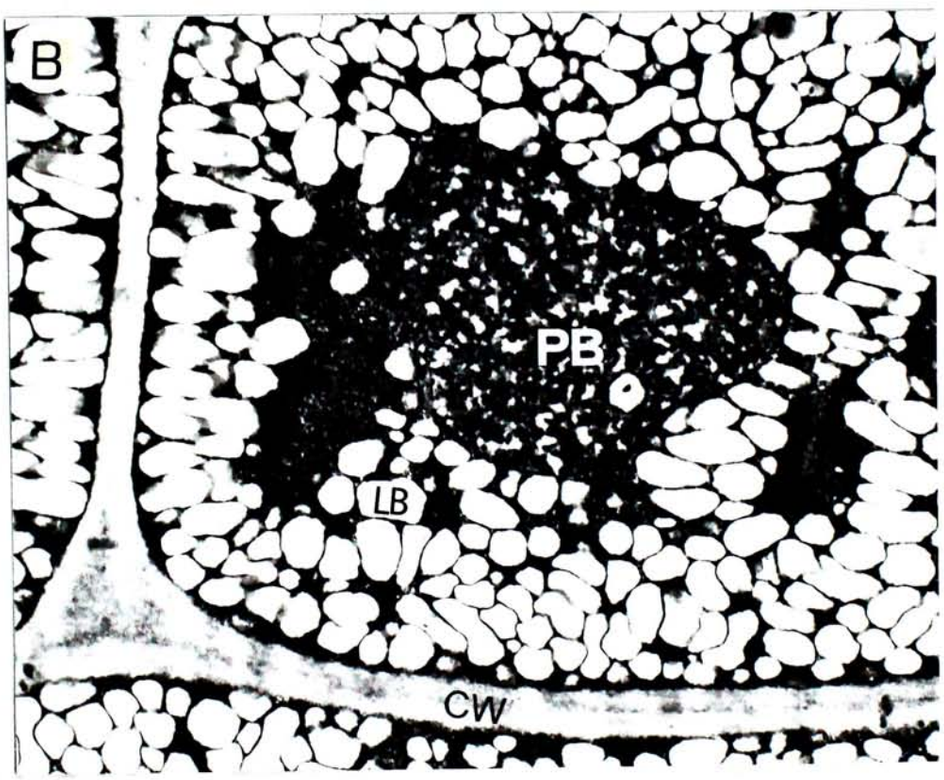


Fig. 4.28 Effect of NCS on hydroxypyruvate reductase activity and growth of excised radish cotyledons. Mean±SD, n=6.

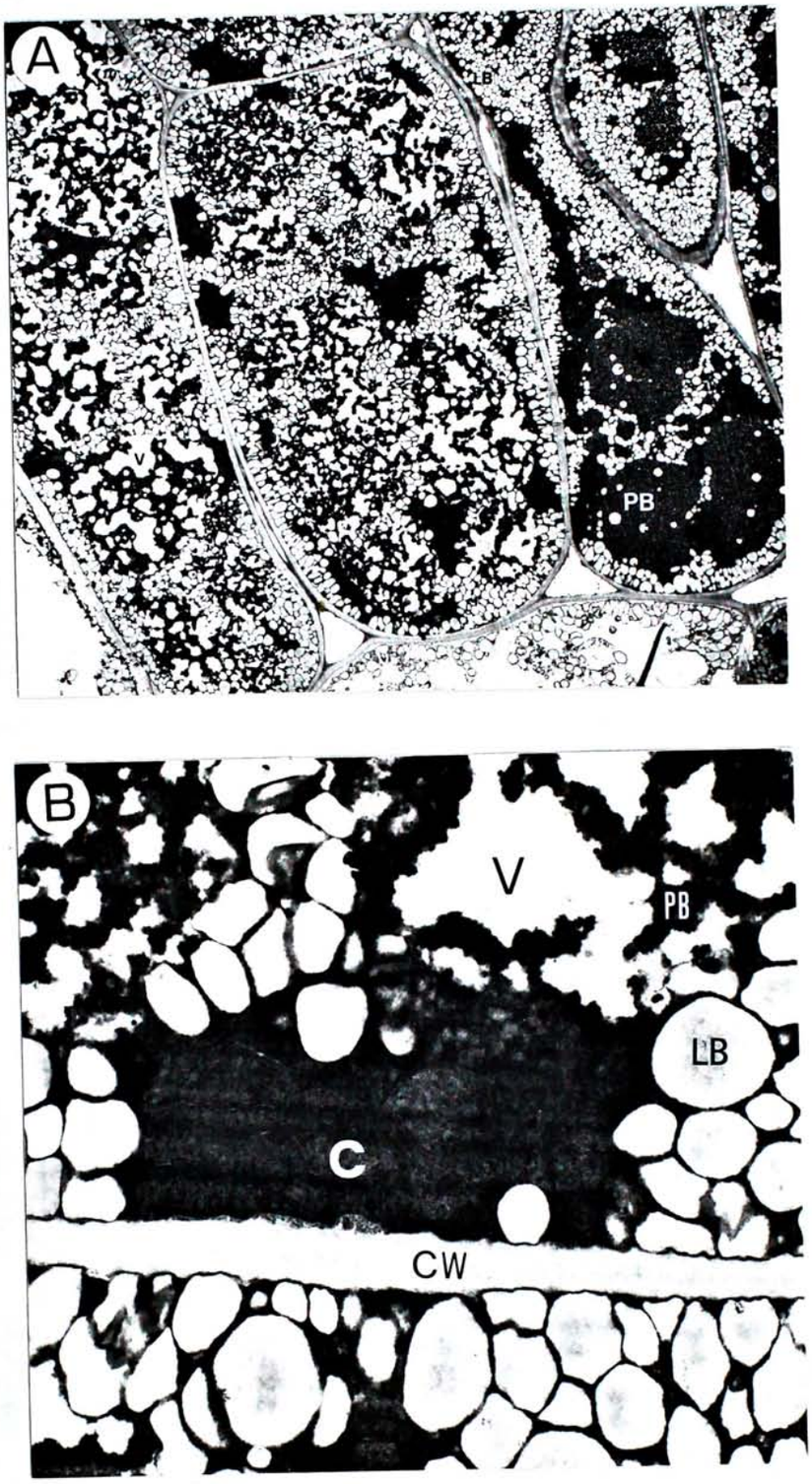
4.5 Effect of NCS on ultrastructural changes of excised radish cotyledons

I. Time course studies

Radish cotyledons were excised from seeds after germination in the dark for 48 hr. Figs. 4.29-4.41 showed that ultrastructural changes of excised cotyledons incubated in different concentrations of NCS (10^{-7} and 10^{-6}M) after exposure to light. As shown in Figs. 4.29A & 4.29B, at the time of excision, lipid bodies were abundant. The protein bodies varied in consistency. Marked degradation of protein and lipid bodies were observed in excised radish cotyledons in the control after 12 hr in the light (Figs. 4.30A & 4.30B), while no chloroplast appeared well developed. NCS (10^{-7} and 10^{-6}M) significantly inhibited the degradation of lipid bodies and protein bodies at 12 hr, there was no chloroplast appeared (Figs. 4.31 & 4.32). At 24 hr, some chloroplasts were appeared in the control. The number of chloroplasts and the degree of their development increased with prolonged incubation in light (Figs. 4.33 & 4.36). However, limited chloroplasts appeared in the excised radish cotyledons treated with 10^{-7}M NCS (Fig. 4.34A). The chloroplasts were formed poorly in NCS-treated cotyledons (Fig. 4.34B). At 48 hr, in the control, a large vacuole and chloroplasts appeared in the center of the cell and near the cell wall, respectively (Fig. 4.39A). The chloroplast appeared well developed (Fig. 4.39B). Similar phenomena were observed in the cotyledons treated with 10^{-7}M NCS (Figs. 4.40A & 4.40B), but in these cotyledons, the number of chloroplast formed was less than the control, the chloroplasts were not as well developed as the control



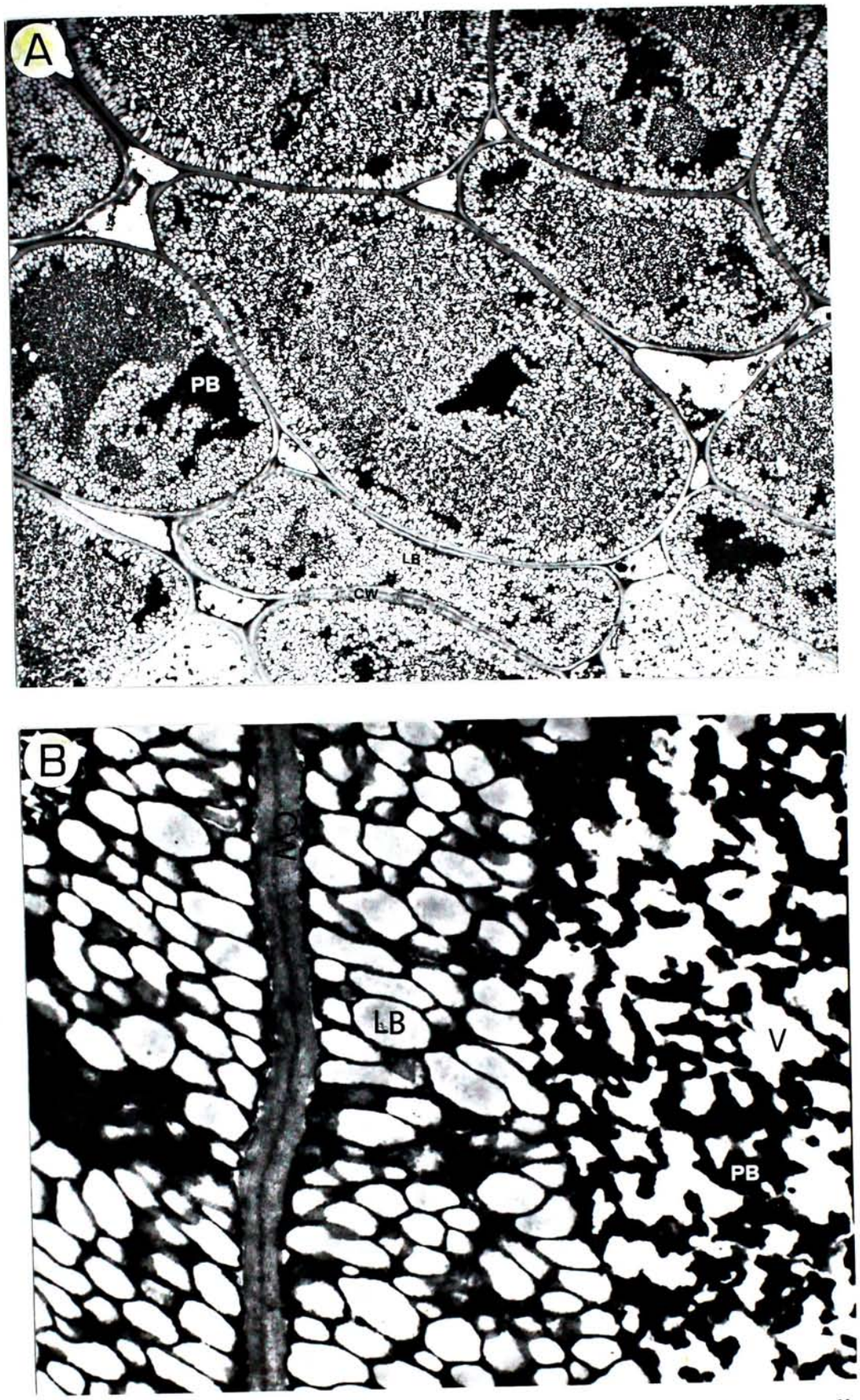
Figs. 4.29A & 4.29B. Electron micrographs of excised radish cotyledonous cells at time of excision. The cotyledons were excised after radish seed germination for 48 hr in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



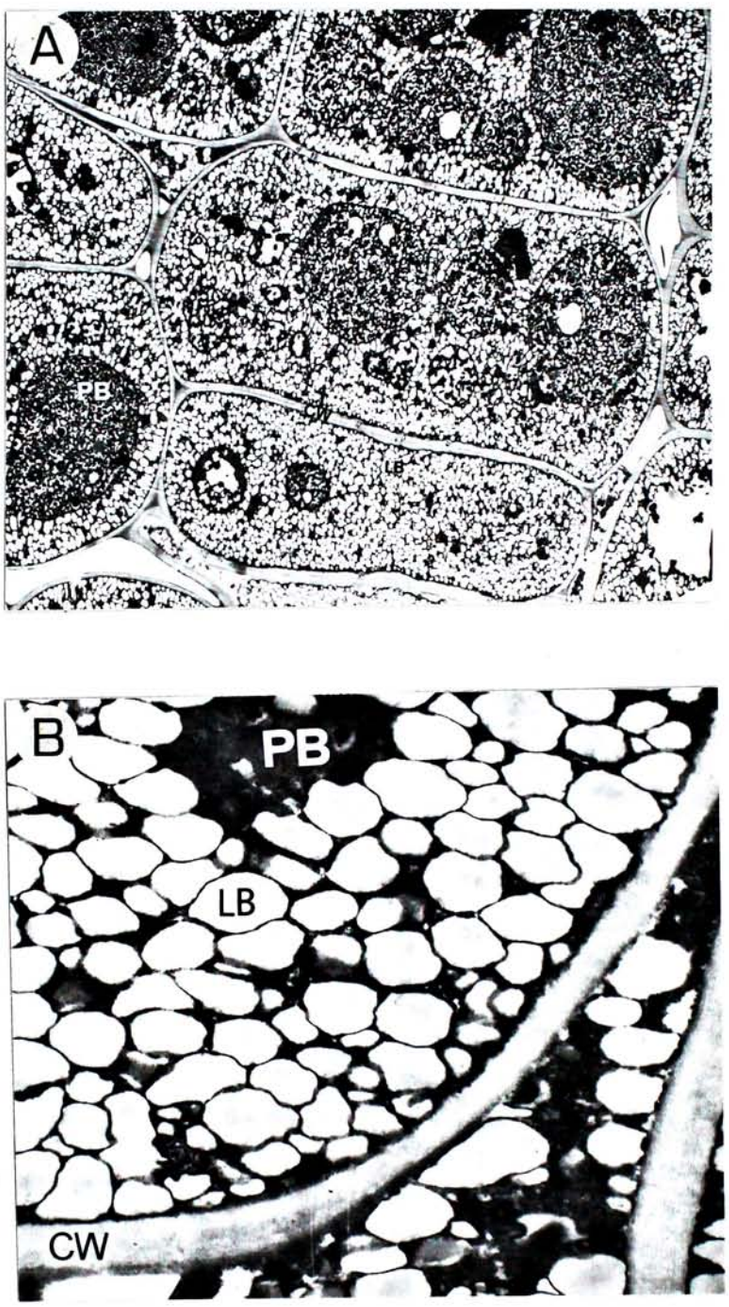
Figs. 4.30A & 4.30B. Electron micrographs of excised radish cotyledonous cells incubated in water for 12 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

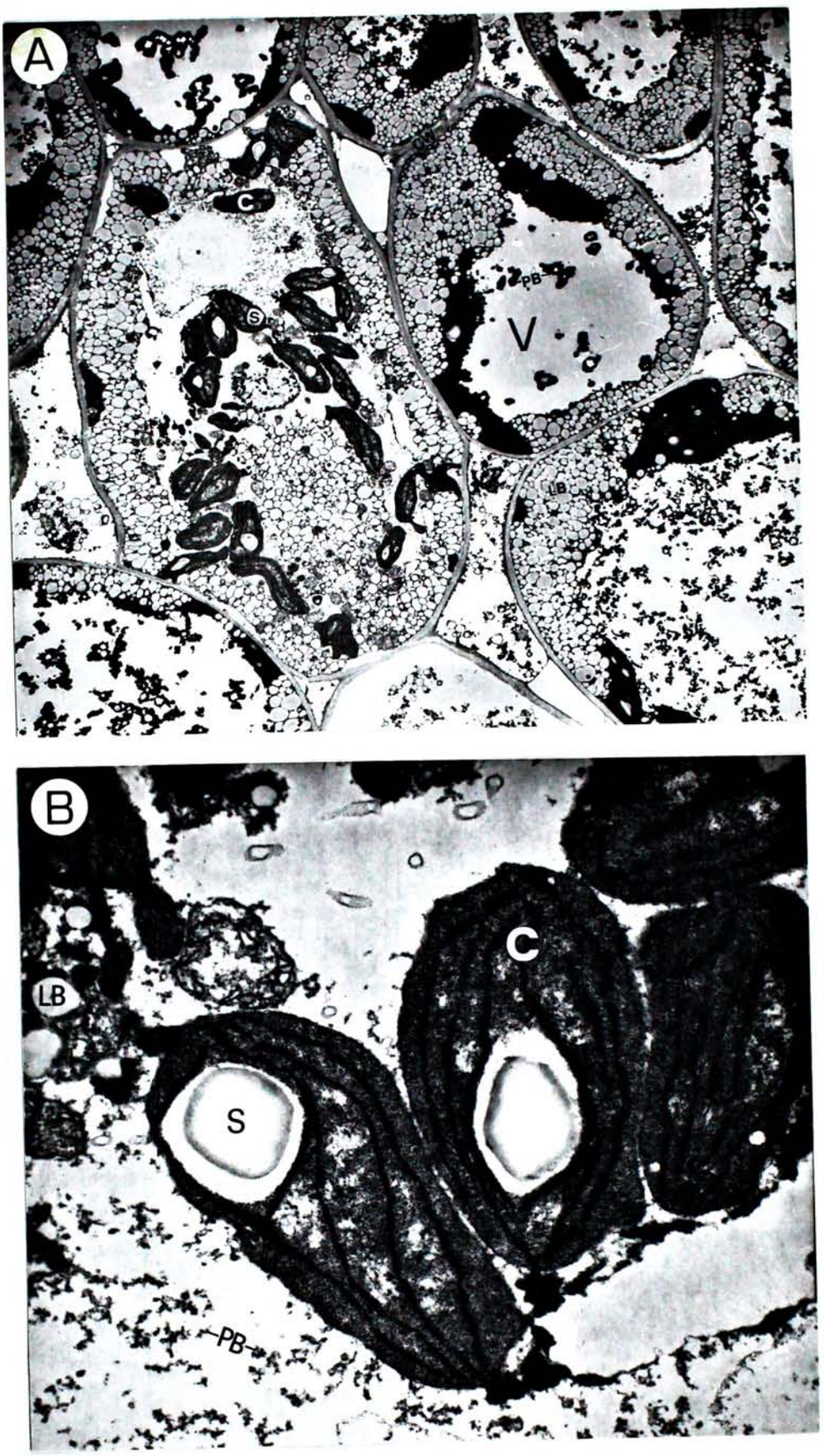
A: low power, x1,800; B: high power, x18,000.



Figs. 4.31A & 4.31B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-7}M NCS for 12 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



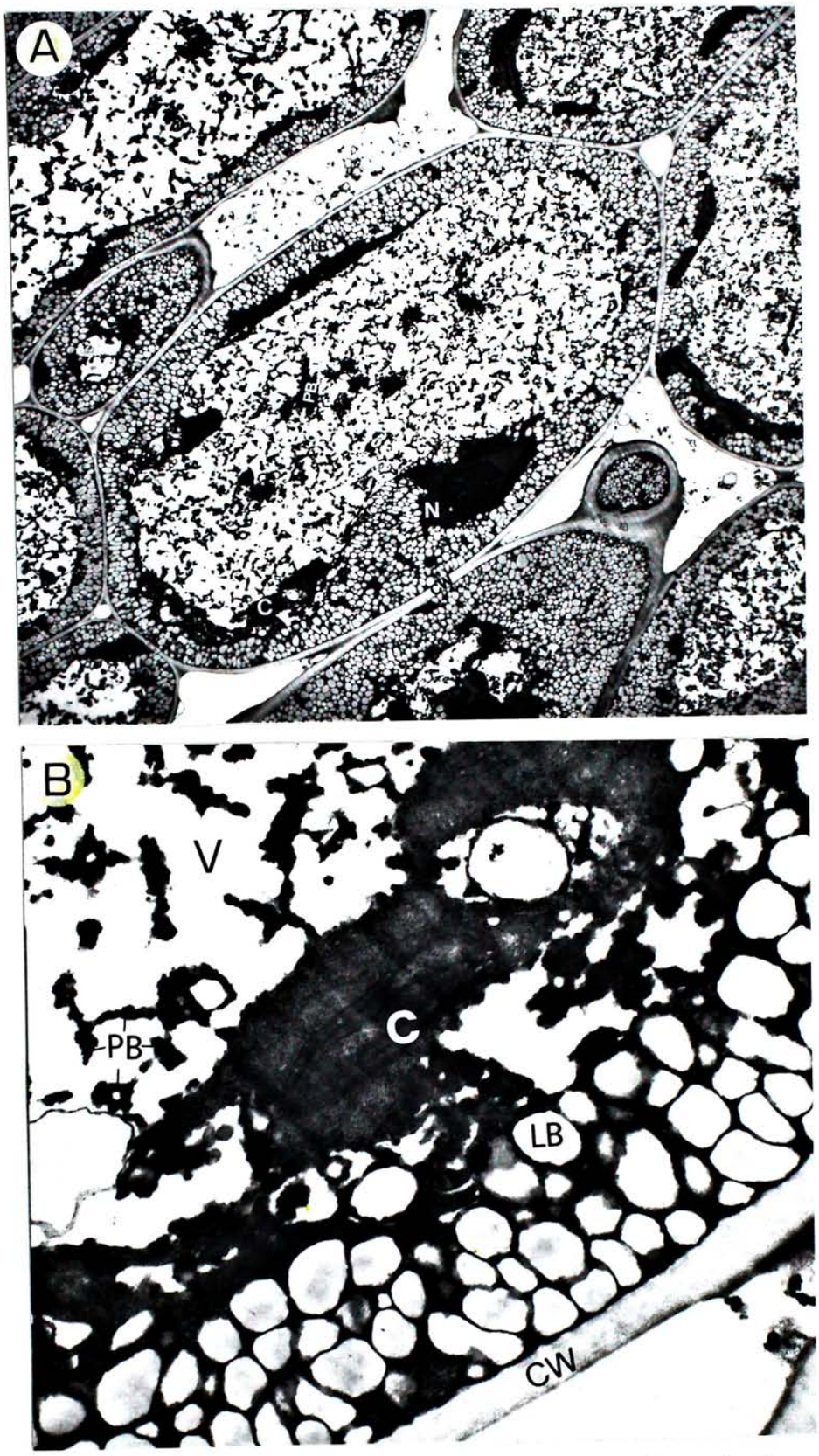
Figs. 4.32A & 4.32B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-6}M NCS for 12 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



Figs. 4.33A & 4.33B. Electron micrographs of excised radish cotyledonous cells incubated in water for 24 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

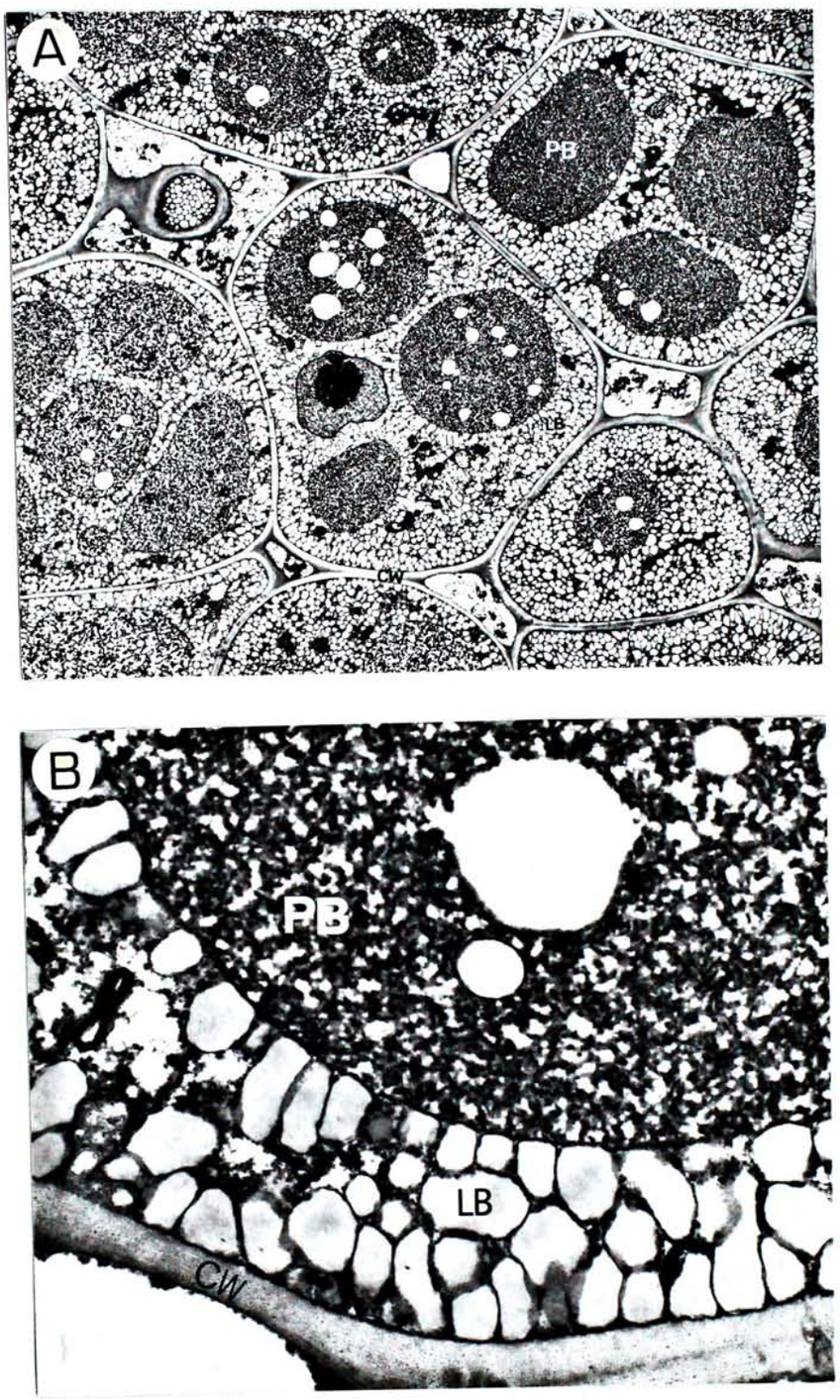
A: low power, x1,800; B: high power, x18,000.



Figs. 4.34A & 4.34B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-7}M NCS for 24 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

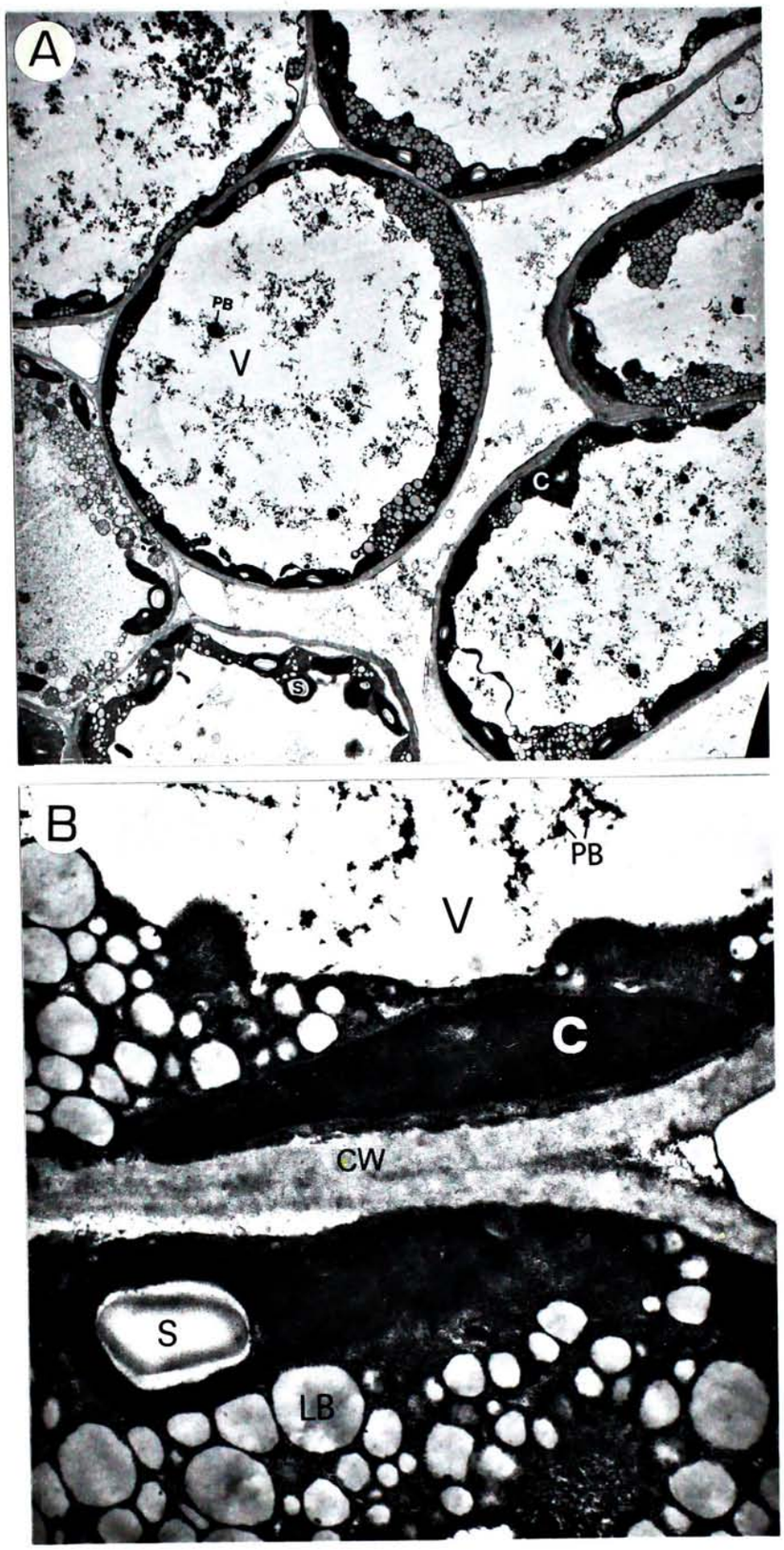
A: low power, x1,800; B: high power, x18,000.



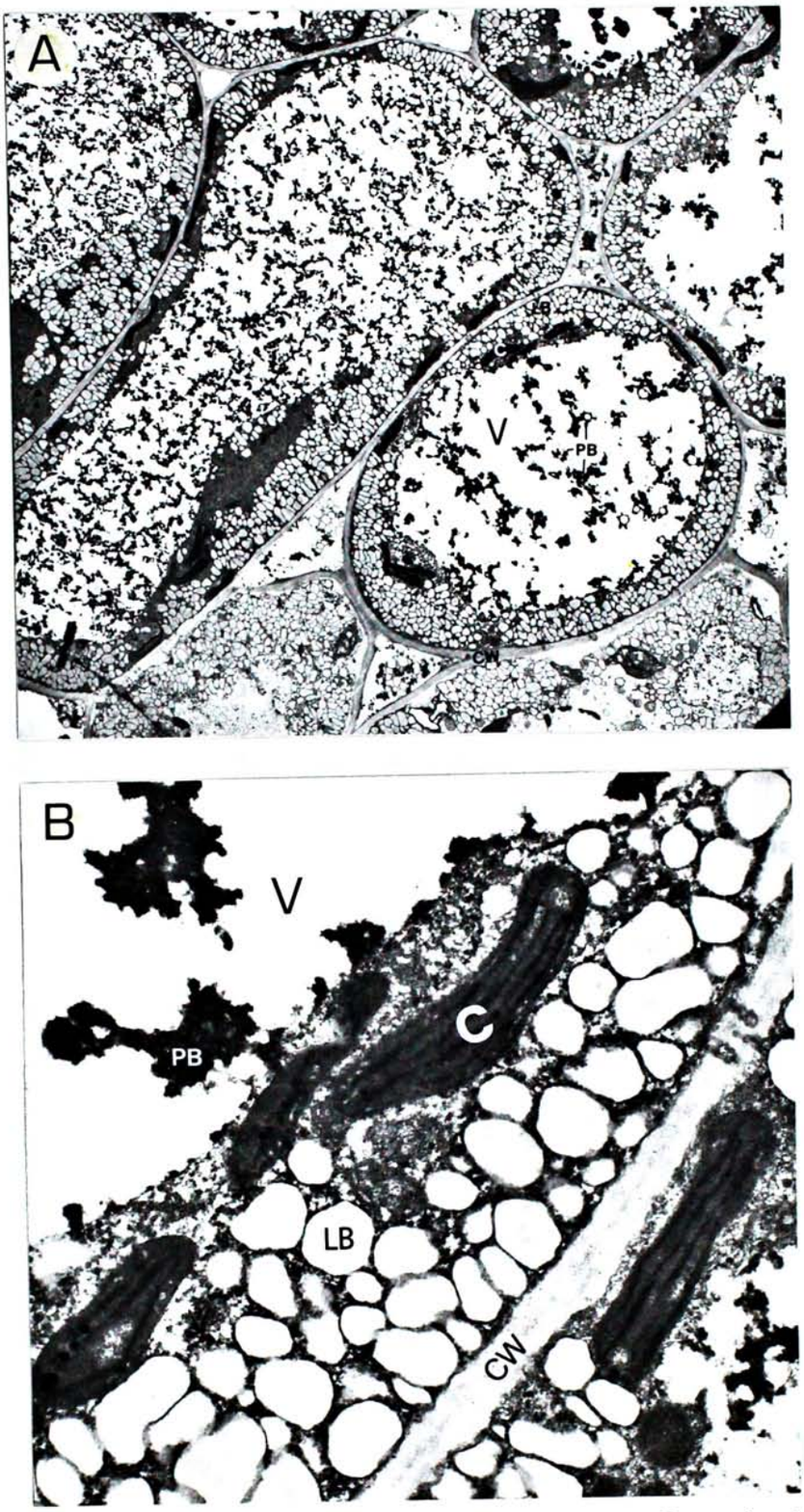
Figs. 4.35A & 4.35B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-6}M NCS for 24 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

A: low power, x1,800; B: high power, x18,000.



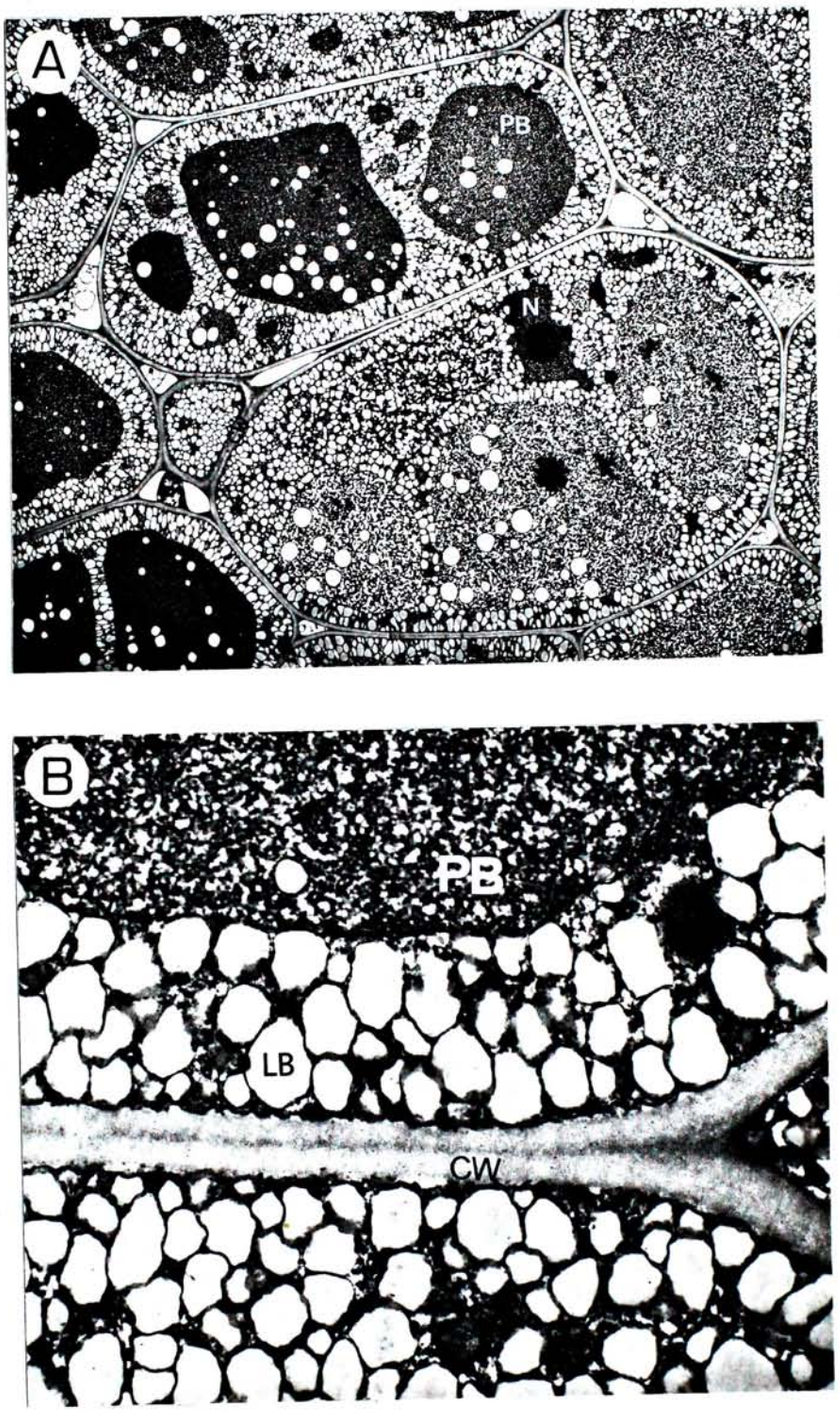
Figs. 4.36A & 4.36B. Electron micrographs of excised radish cotyledonous cells incubated in water for 36 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



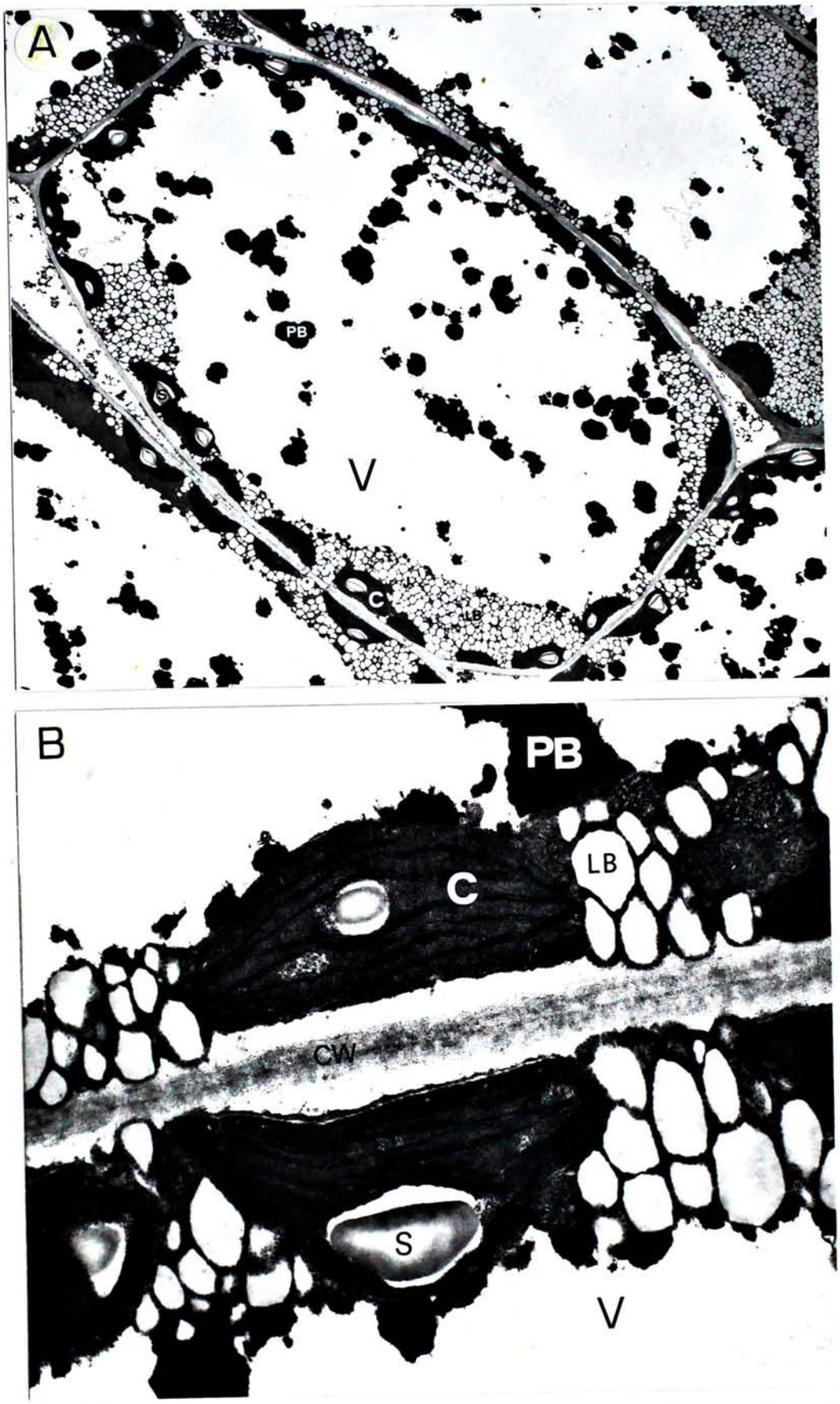
Figs. 4.37A & 4.37B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-7}M NCS for 36 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

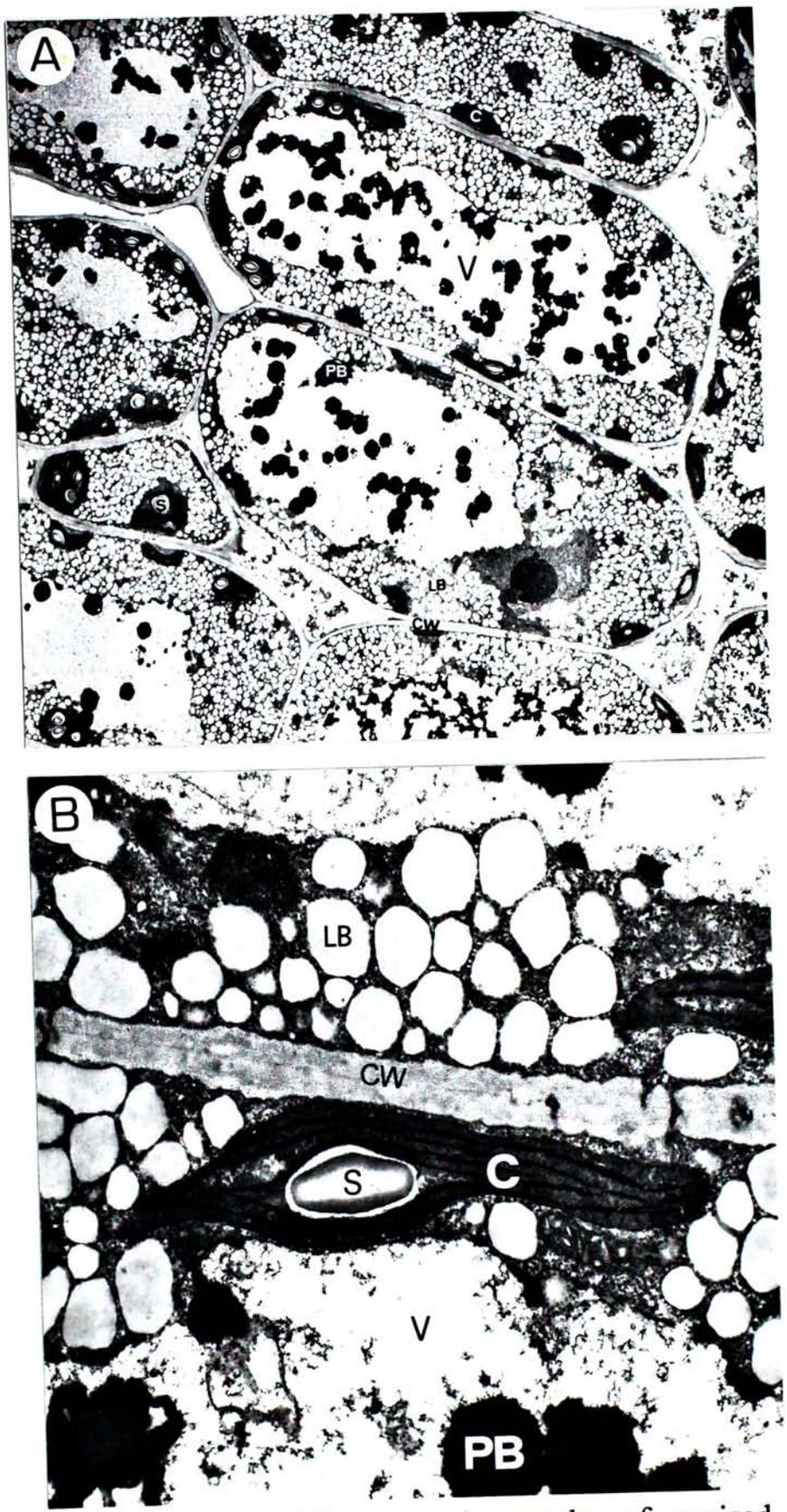
A: low power, x1,800; B: high power, x18,000.



Figs. 4.38A & 4.38B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-6}M NCS for 36 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



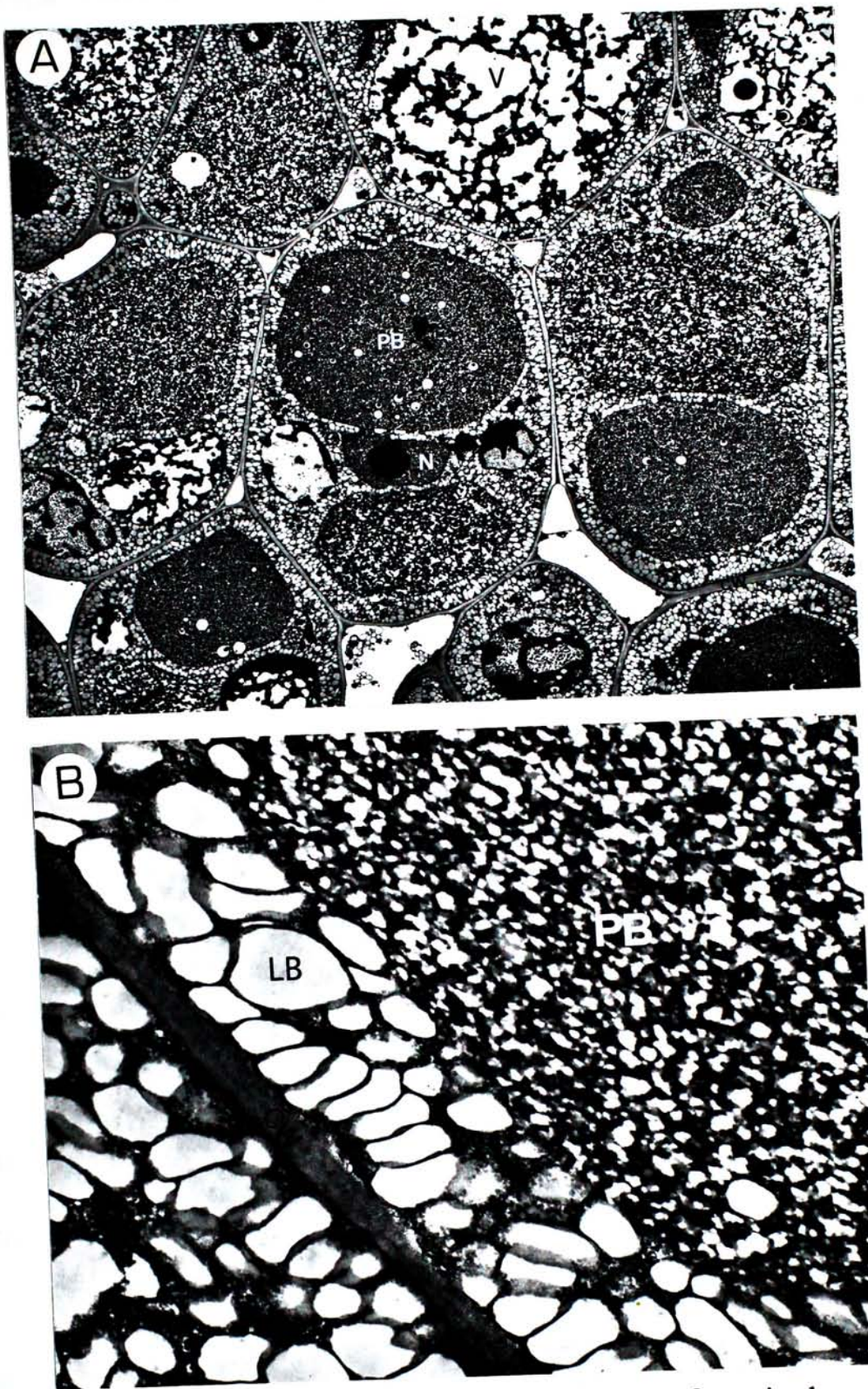
Figs. 4.39A & 4.39B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



Figs. 4.40A & 4.40B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-7}M NCS for 48 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

A: low power, x1,800; B: high power, x18,000.



Figs. 4.41A & 4.41B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-6}M NCS for 48 hr in light at 28°C. Excised radish cotyledons were obtained after germinating for 48 hr in dark at 22±2°C.

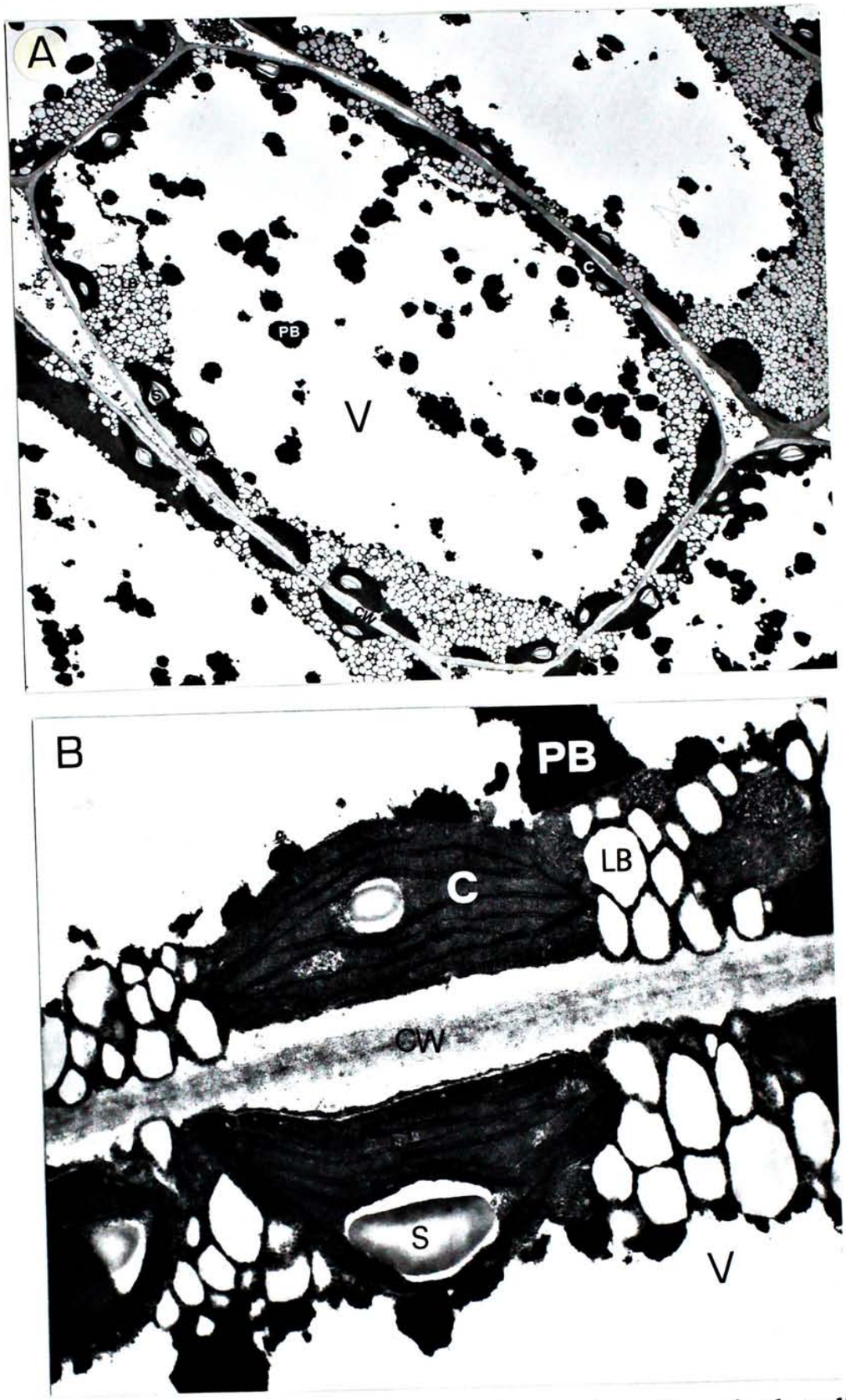
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

A: low power, x1,800; B: high power, x18,000.

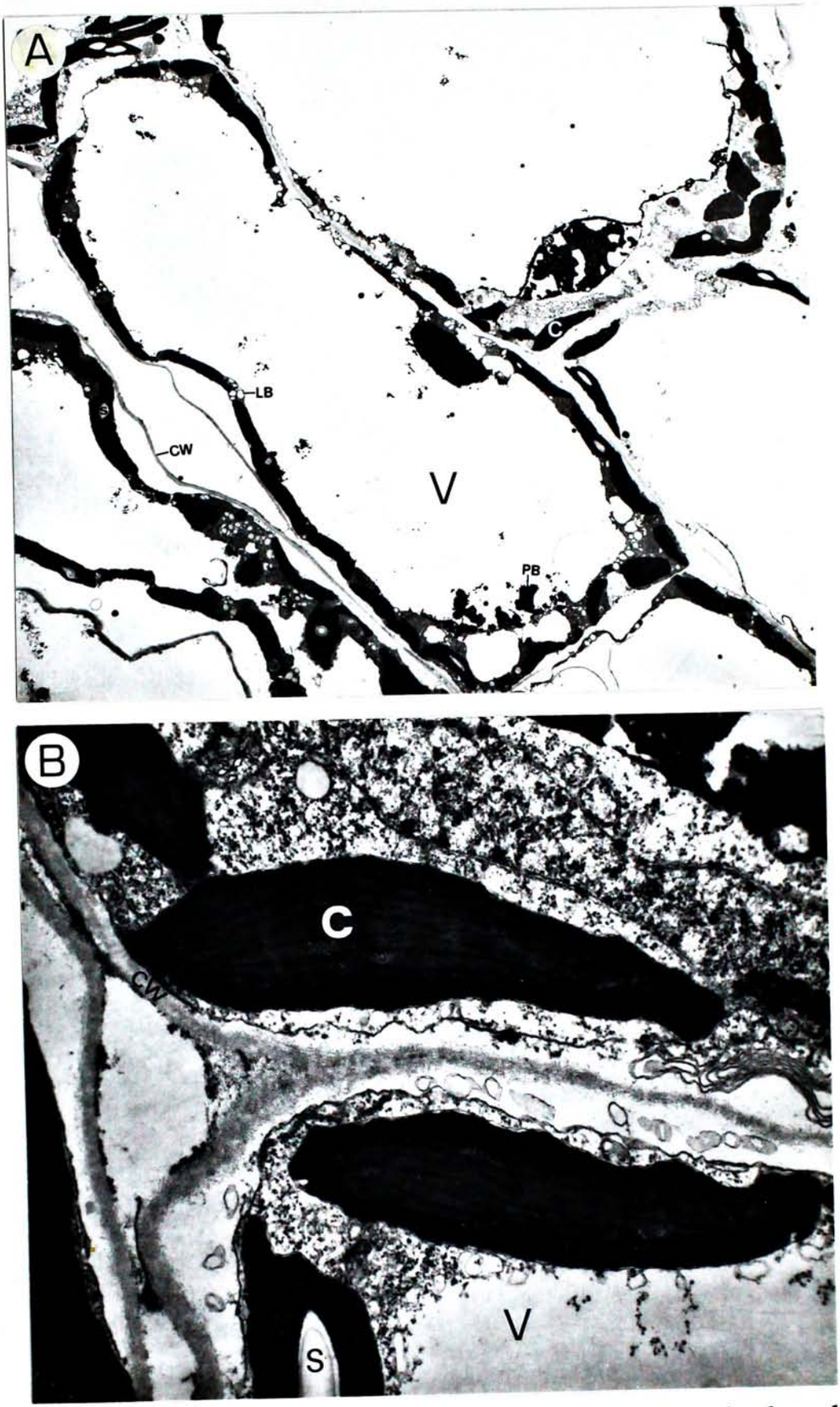
cotyledons (Figs. 4-30B & 4.40B). NCS (10^{-6}M) completely blocked the degradation of lipid bodies and protein bodies, as well as the chloroplast development of excised radish cotyledons during incubation for 48 hr in light (Figs 4.32, 4.35, 4.38 and 4.41).

II. Effect of NCS, BA and ABA on the ultrastructural change of excised radish cotyledon in light

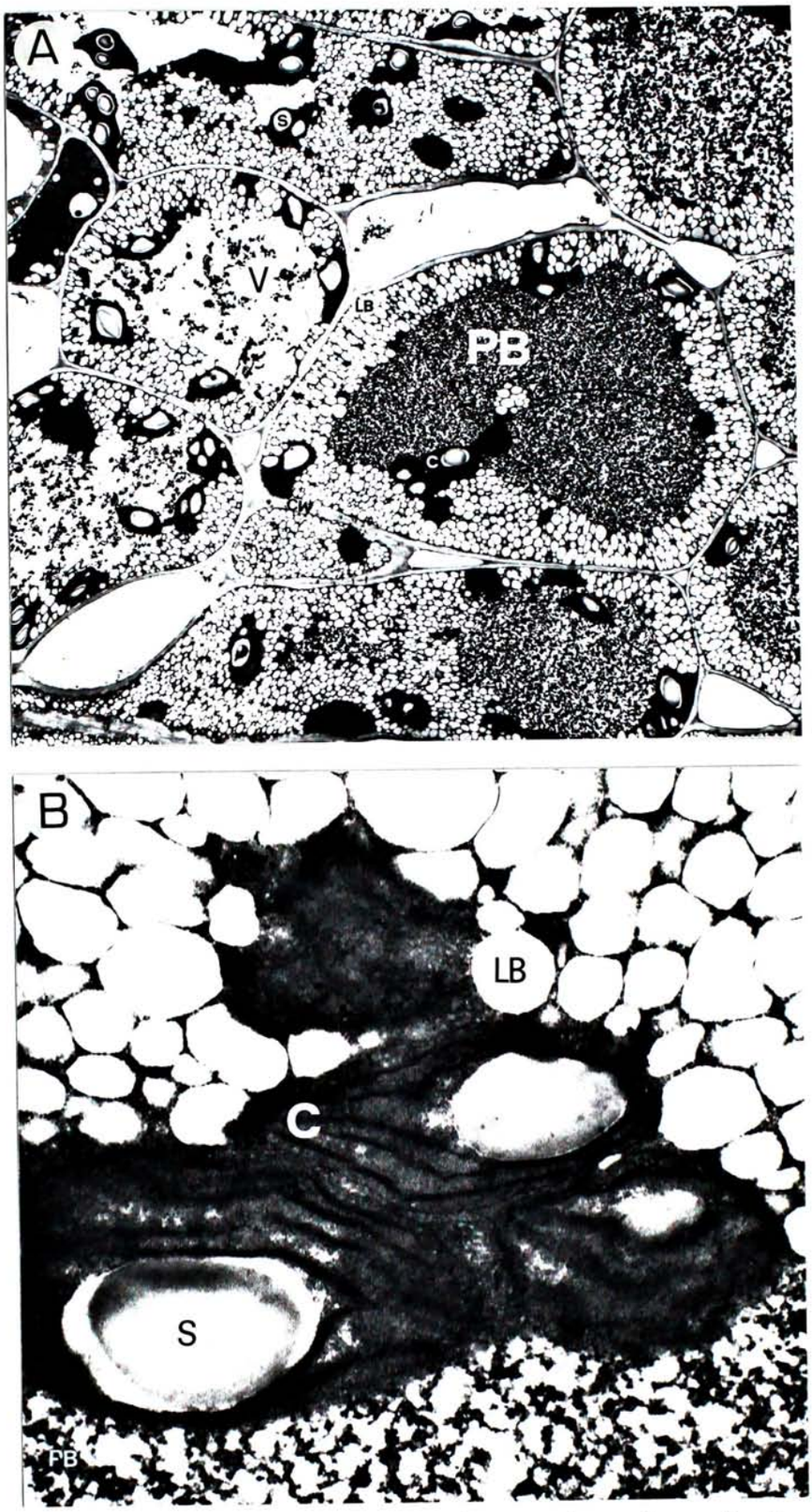
As shown in Fig. 4.42, the significant ultrastructural changes in excised radish cotyledons occurred at 48 hr in light after excision. In BA-treated cotyledons, BA appeared to have stimulated the breakdown of protein bodies and lipid bodies (Figs. 4.43A & 4.43B) as compared to control cotyledons (Figs. 4.42A & 4.42B). Chloroplasts in BA-treated and water control cotyledons were well developed. In ABA-treated cotyledons (Figs. 4.44A & 4.45A), ABA appeared to inhibit protein bodies and lipid bodies degradation as compared to the control ones. The number of chloroplasts was also blocked by ABA and decreased with increasing concentrations of ABA. The formed chloroplasts were not well developed (Figs. 4.44B & 4.45B). Figs. 4.46-4.48 showed that NCS was more effective in the inhibition of protein bodies and lipid bodies degradation than ABA. NCS (10^{-6} and 10^{-5}M) completely blocked protein bodies and lipid bodies degradation (Fig. 4.47A). In 10^{-7}M NCS-treated cotyledons, some chloroplasts were found, but the extent of the development of these chloroplasts was not as well developed as the control cotyledons (Figs. 4.42B & 4.46B). However, in the 10^{-6} and 10^{-5}M NCS-treated cotyledons, there were no chloroplast formations (Figs. 4.47B & 4.48B).



Figs. 4.42A & 4.42B. Electron micrographs of excised radish cotyledonous cells after incubation in water for 48 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



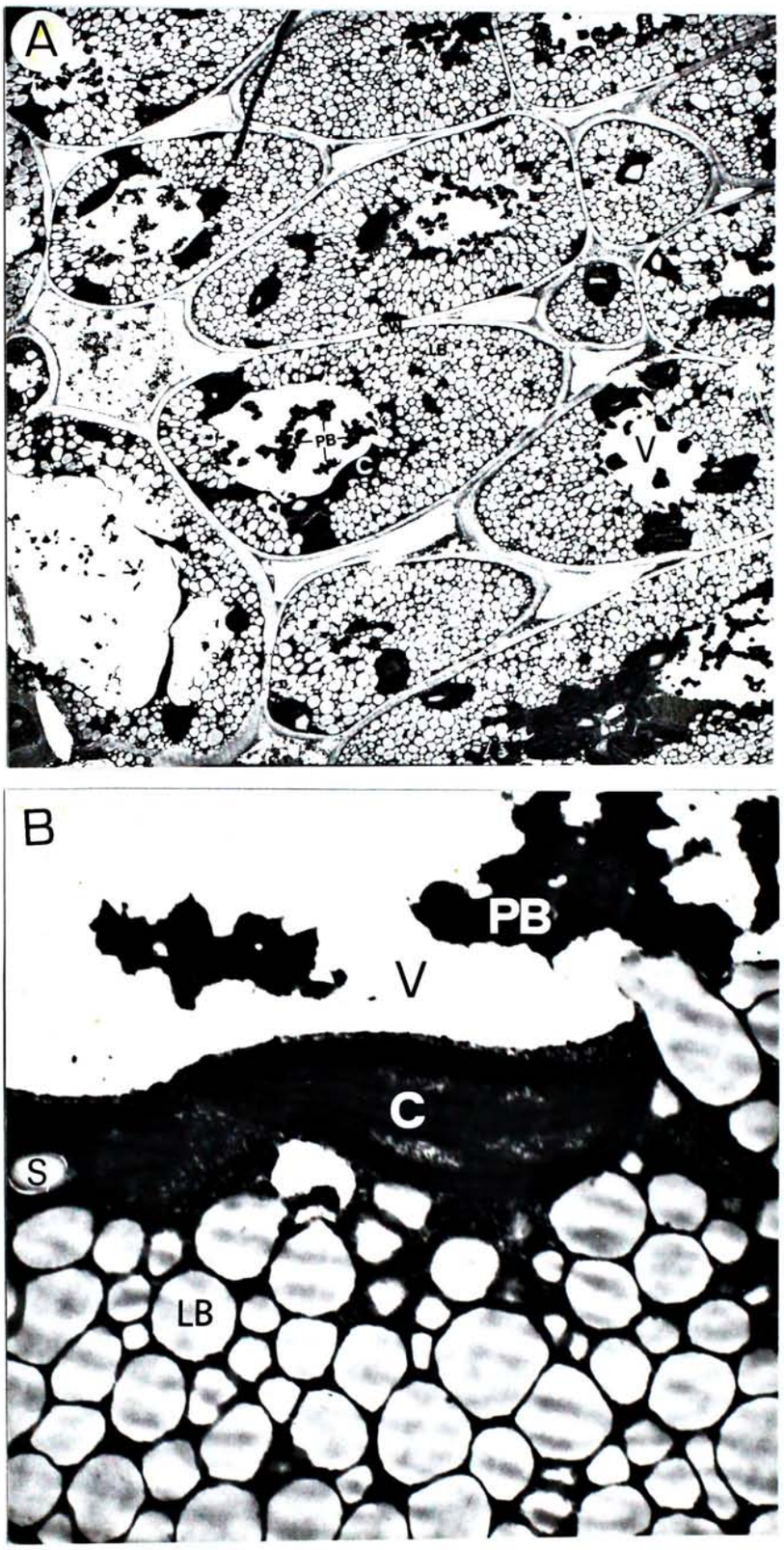
Figs. 4.43A & 4.43B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-5}M BA for 48 hr in light at 28°C.
Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



Figs. 4.44A & 4.44B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-6}M ABA for 48 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

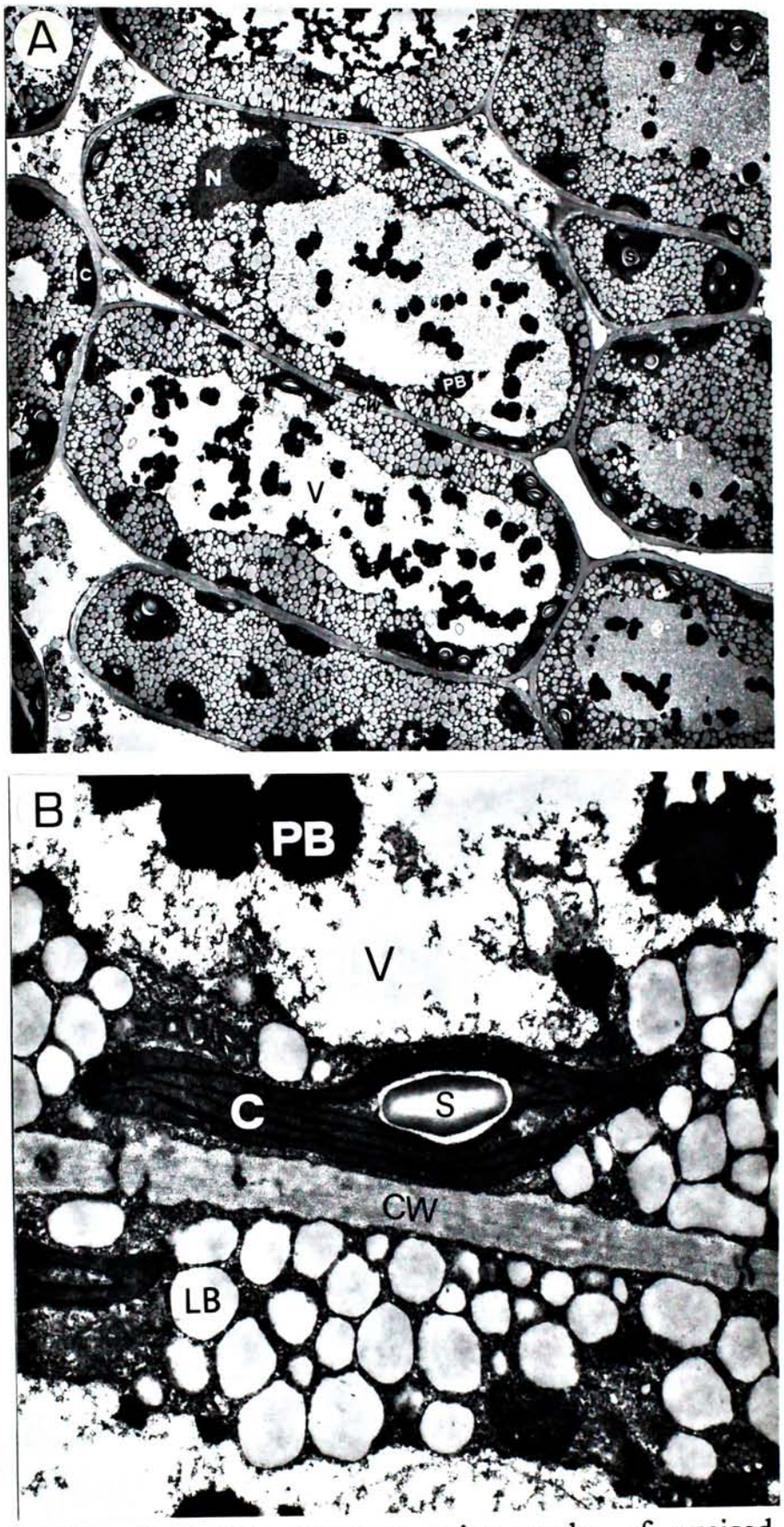
A: low power, x1,800; B: high power, x18,000.



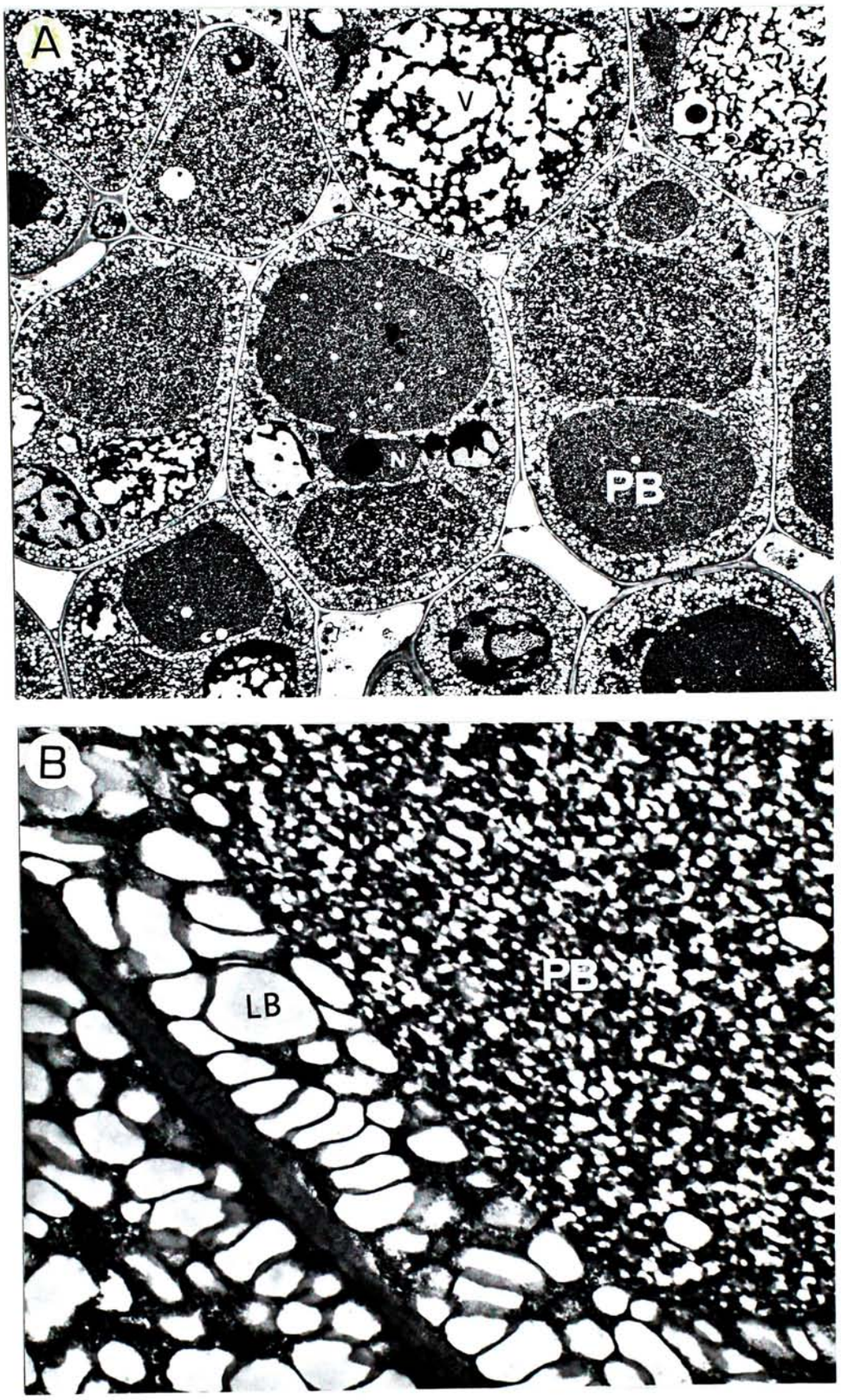
Figs. 4.45A & 4.45B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-5}M ABA for 48 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.

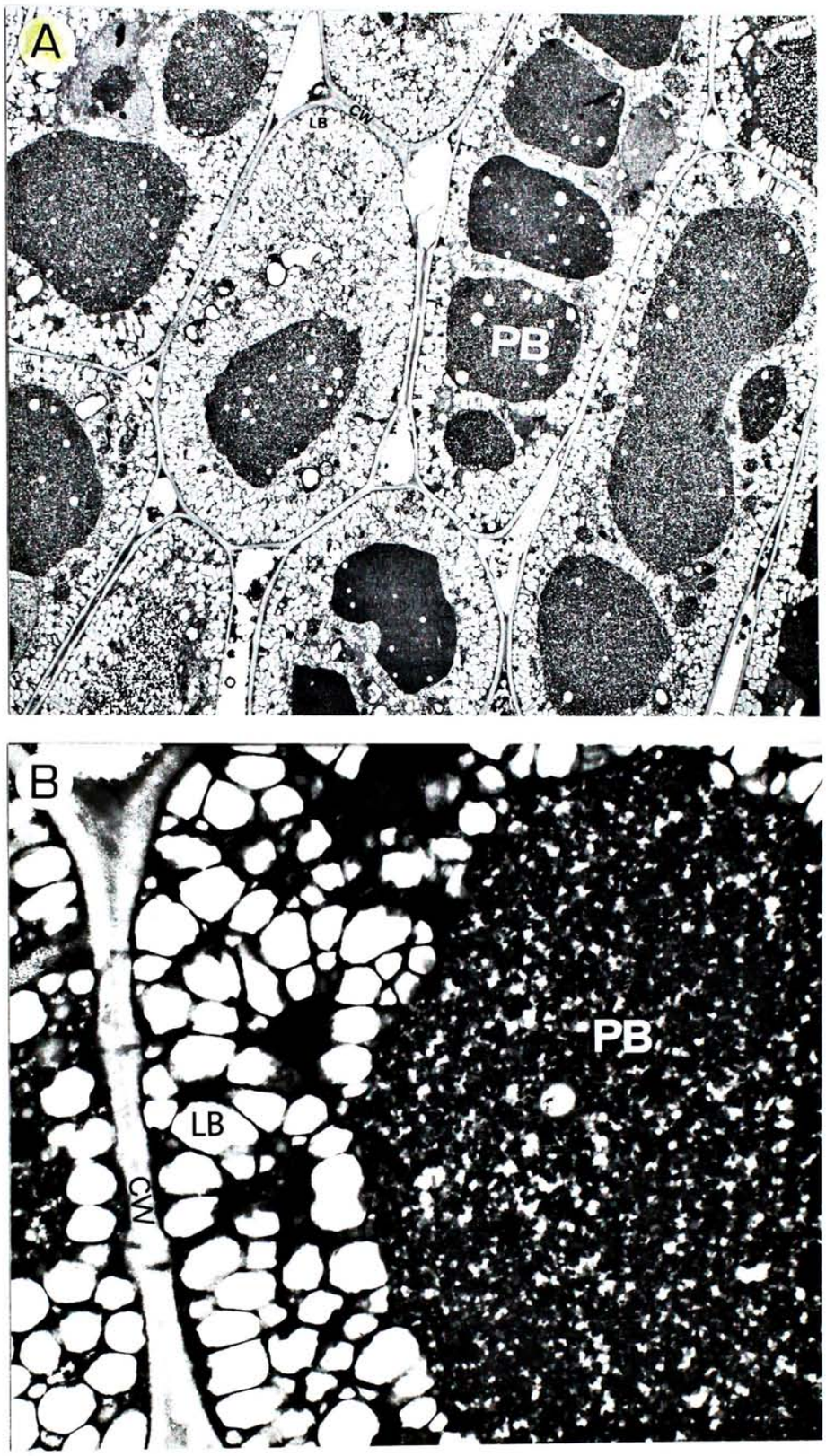
A: low power, x1,800; B: high power, x18,000.



Figs. 4.46A & 4.46B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-7}M NCS for 48 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



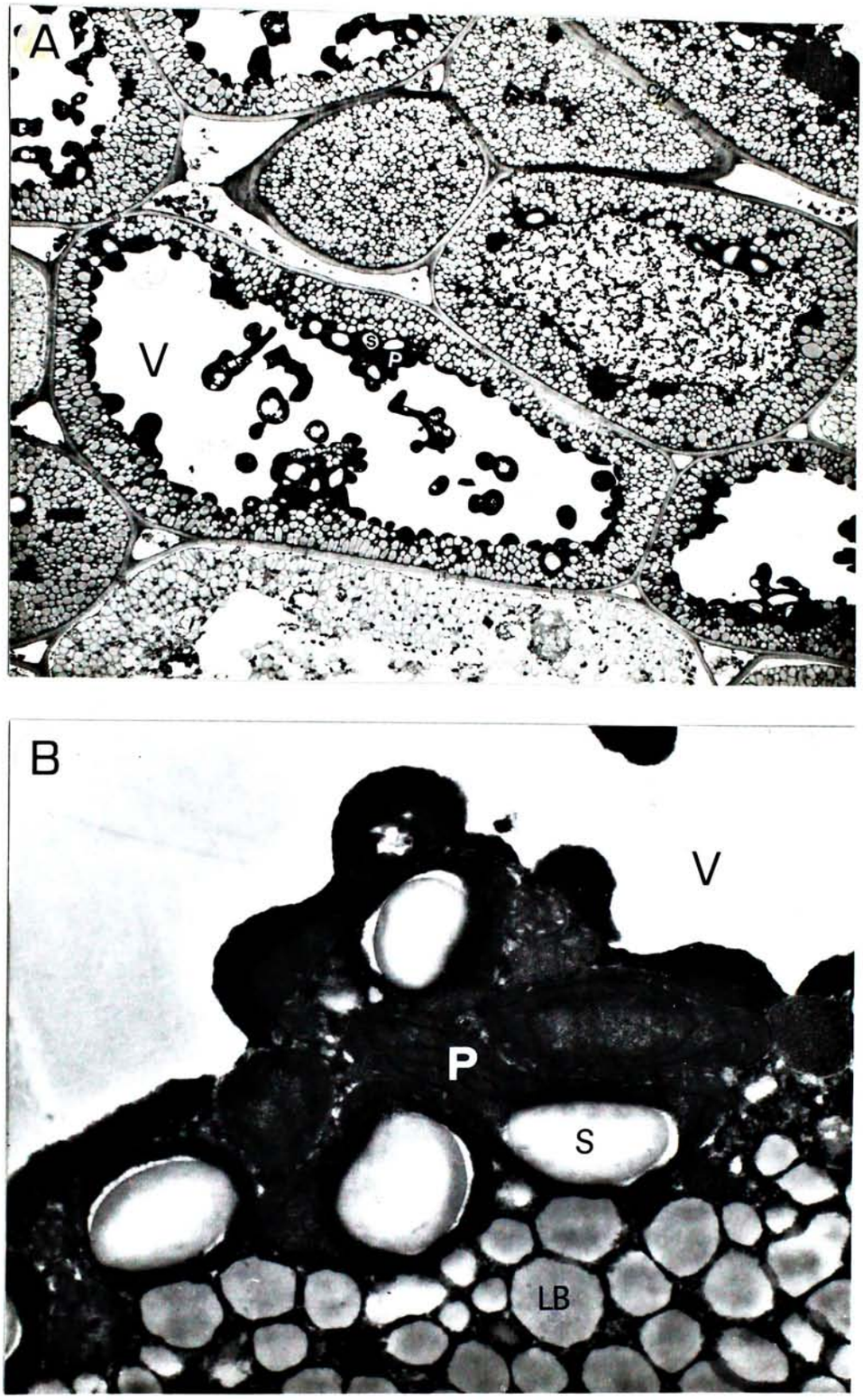
Figs. 4.47A & 4.47B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-6}M NCS for 48 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



Figs. 4.48A & 4.48B. Electron micrographs of excised radish cotyledonous cells treated with 10^{-5}M NCS for 48 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.

III. Effect of a pretreatment with dark on the inhibition of NCS on ultrastructural change of excised radish cotyledons in light

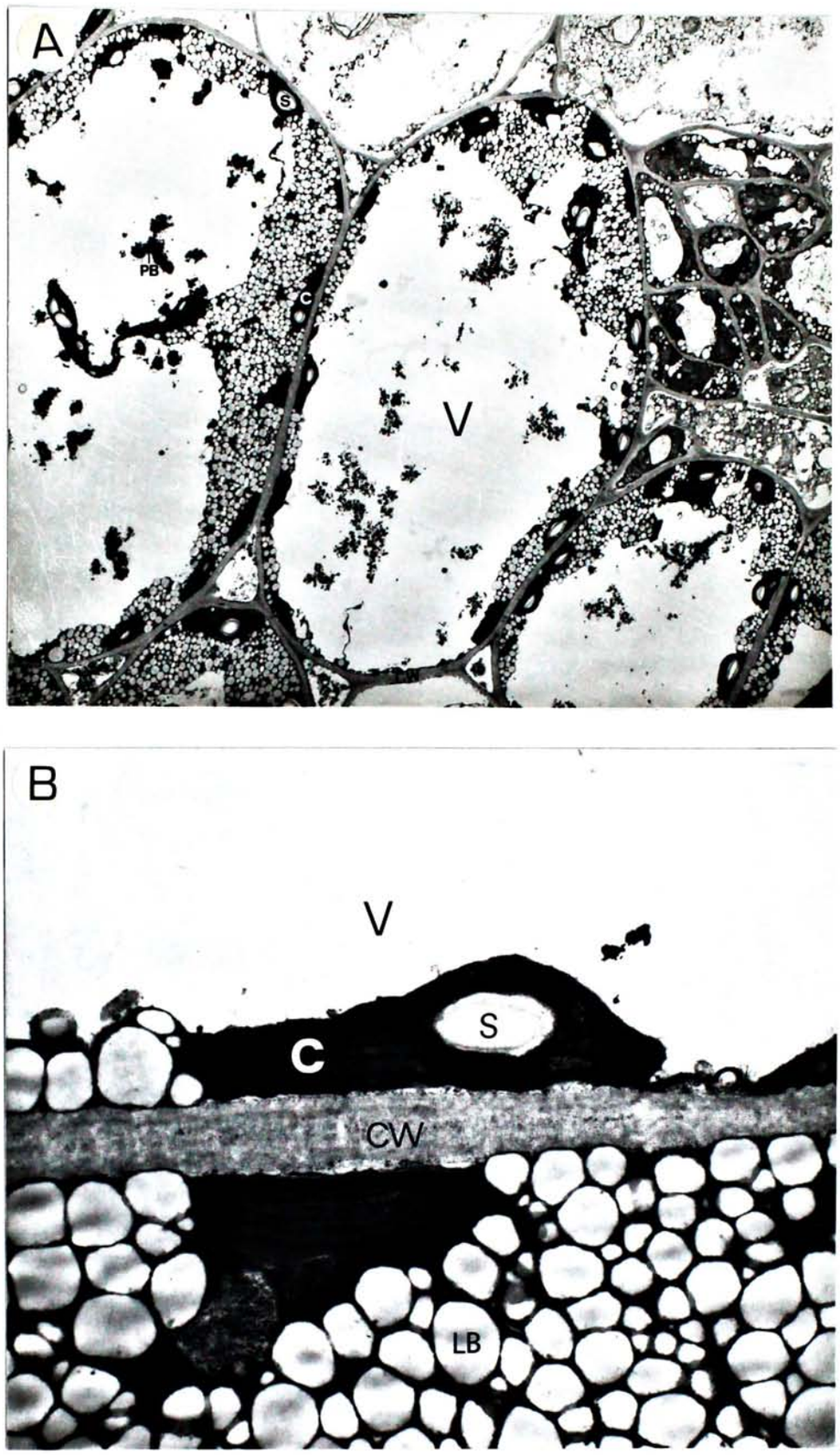
Excised radish cotyledons, which were incubated in distilled water for 48 hr in the dark, showed significant degradation of protein and lipid bodies in the cells (Fig. 4.49A). As Fig. 4.49B illustrated, the etioplasts with large starch appeared in the cells of excised cotyledon and a few plastid membranes mostly appeared in the center of cells. After 12 hr exposure to light, however, the plastid membrane had developed and grana had formed in the cells of cotyledon. They were located in the periphery of the cell (Fig. 4.50B). Similar phenomena were observed in 10^{-7}M NCS-treated cells (Fig. 4.51B). But the level of formed grana in the plastids was not very well developed as compared to the control cells, and only a part of plastids appeared near the periphery of the cell. In the cells treated with 10^{-6}M or 10^{-5}M concentrations of NCS, there were hardly any plastids developing or grana forming, and they only exhibited the degradation of lipid bodies and protein bodies progressively, which decreased as concentrations of NCS increased (Figs. 4.52B & 4.53B). At 24 hr exposure to light, the chloroplasts either in the control cells or in the NCS-treated (10^{-7}M) cells were well developed (Figs. 4.54B & 4.55B). However, in NCS-treated cells (10^{-6}M or 10^{-5}M), the plastid membrane showed almost no further development and no grana formation (Figs. 4.56B & 4.57B).



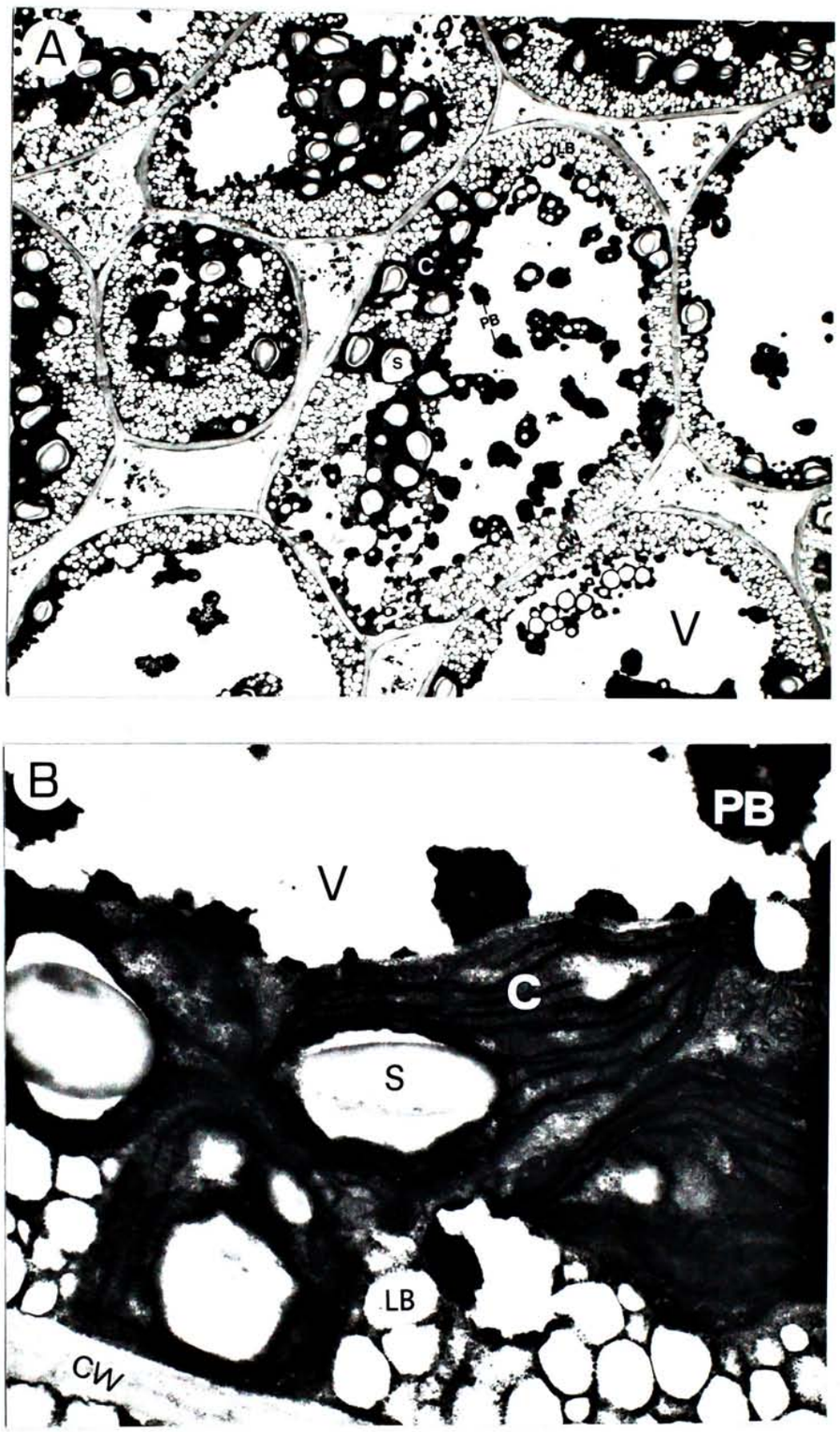
Figs. 4.49A & 4.49B. Electron micrographs of excised radish cotyledonous cells after incubation for 48 hr in dark at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.

C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.

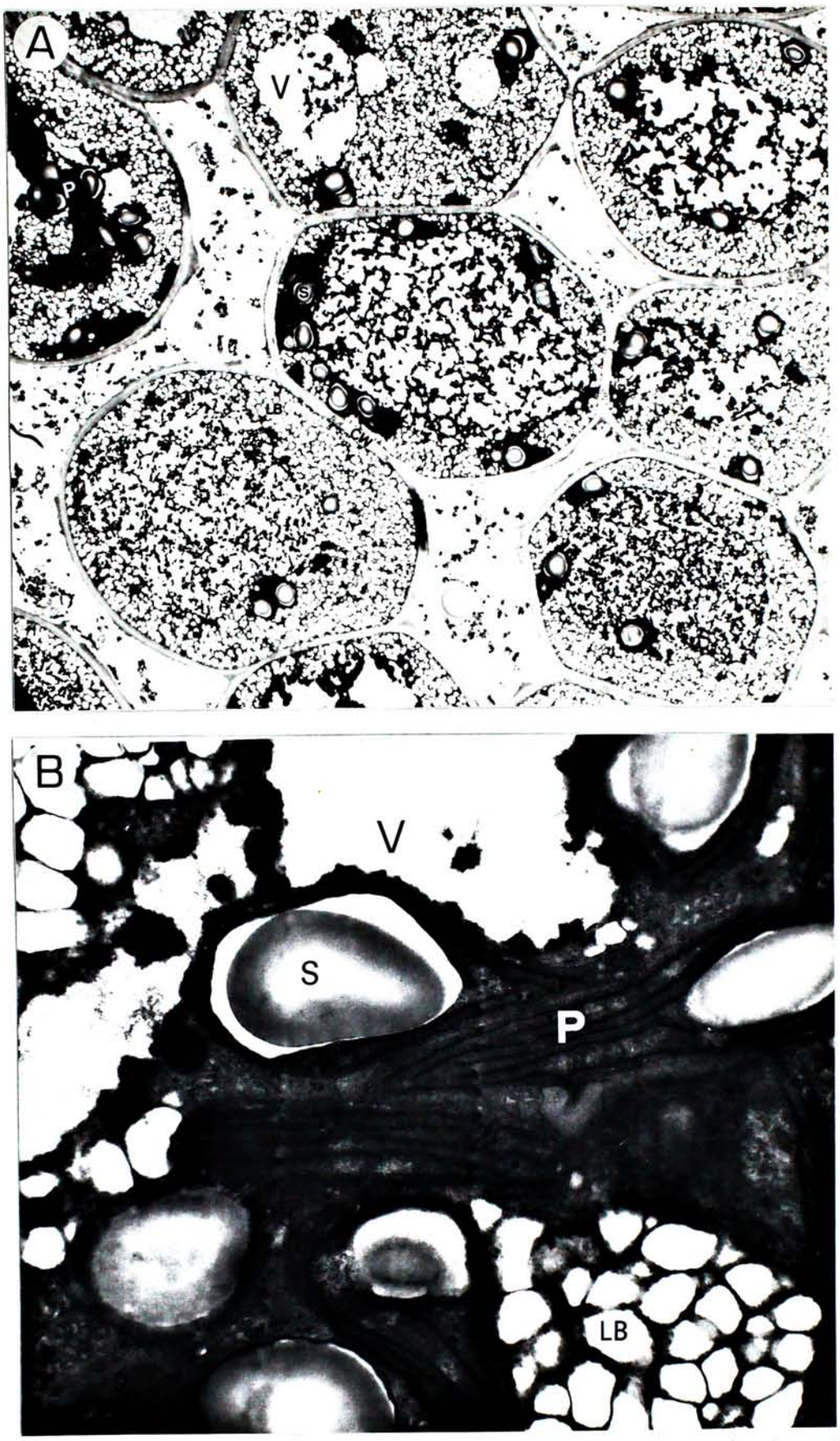
A: low power, x1,800; B: high power, x18,000.



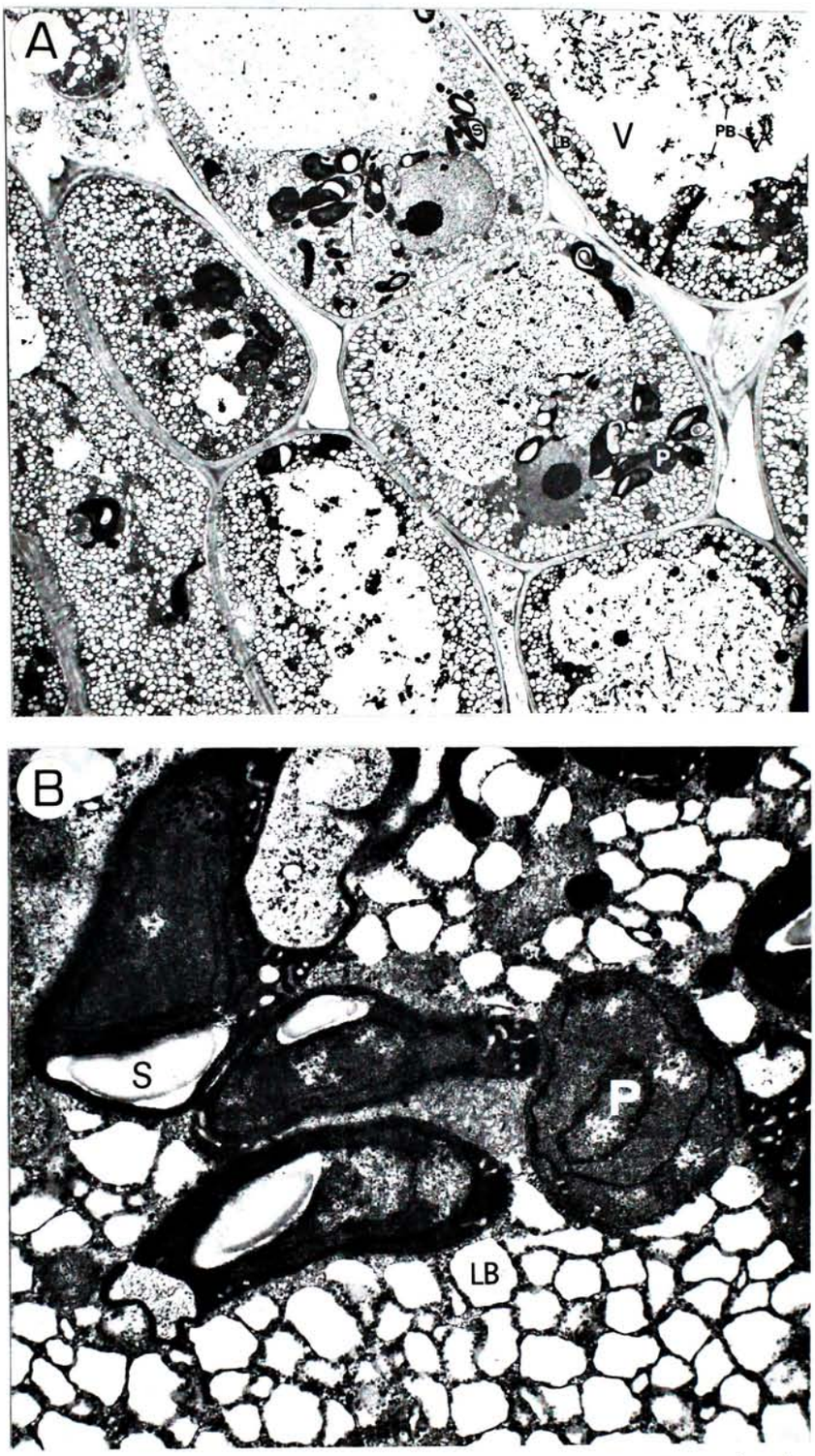
Figs. 4.50A & 4.50B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in dark and for 12 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



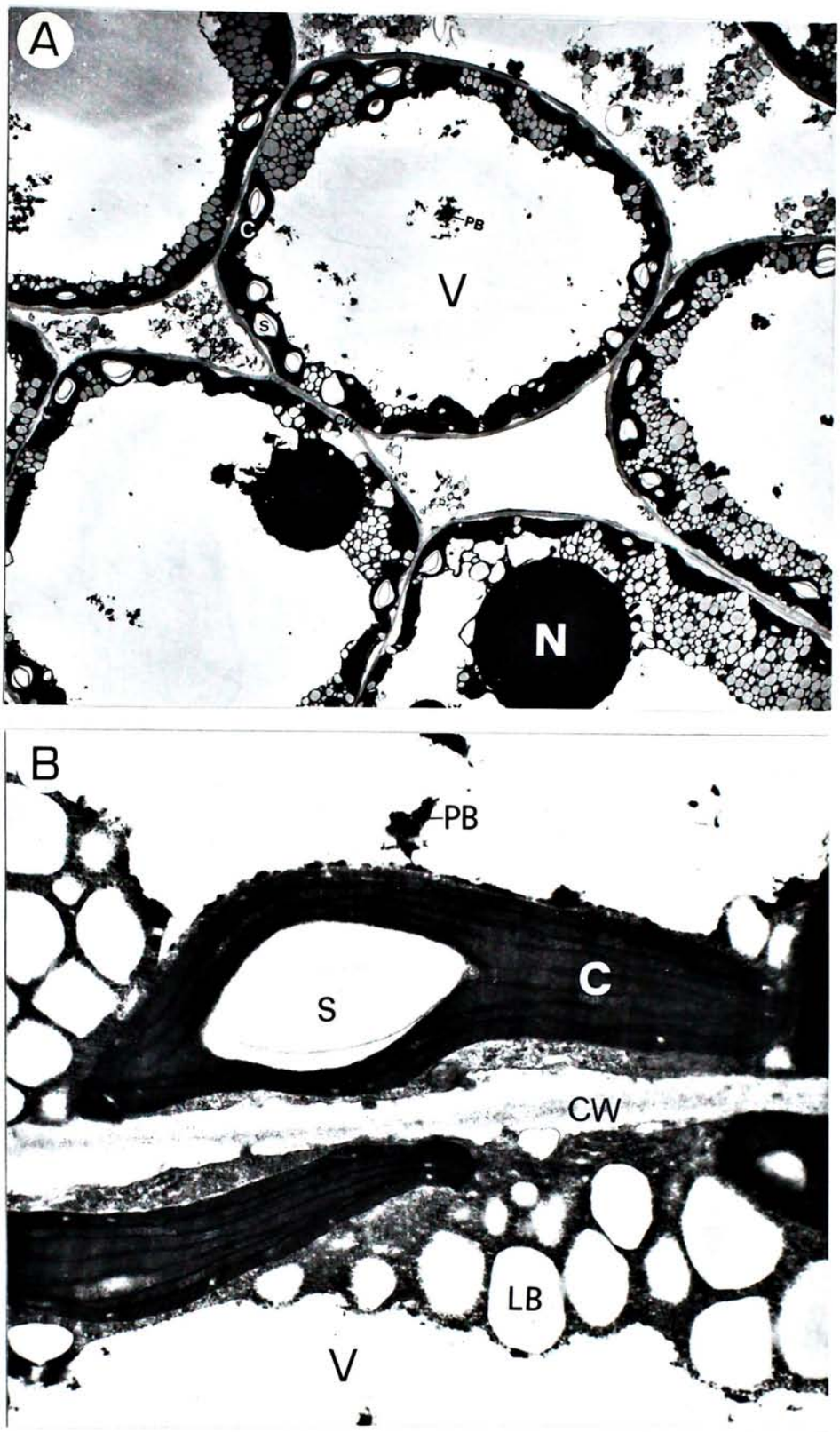
Figs. 4.51A & 4.51B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in dark and in 10^{-7}M NCS for 12 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



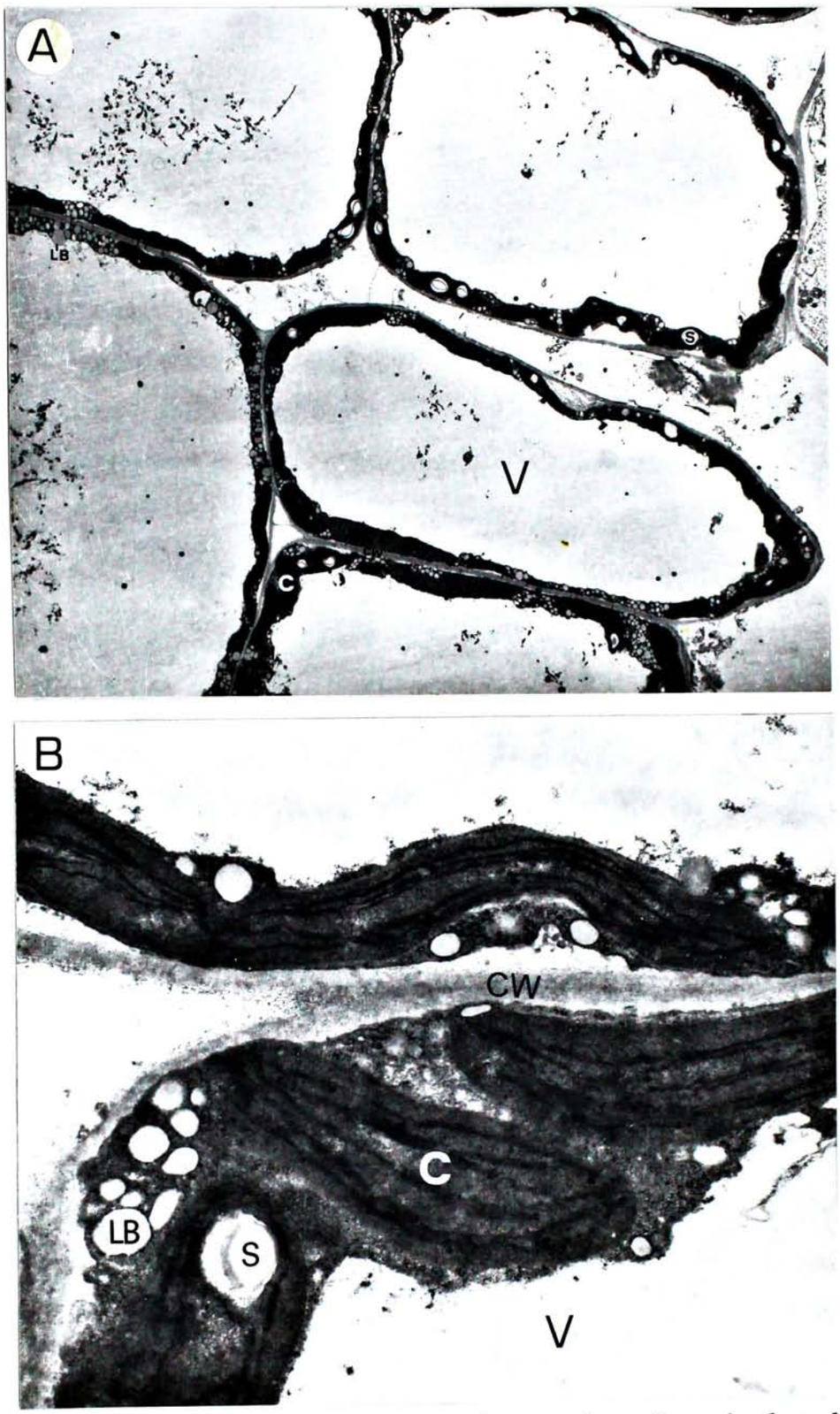
Figs. 4.52A & 4.52B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in dark and in 10^{-6}M NCS for 12 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



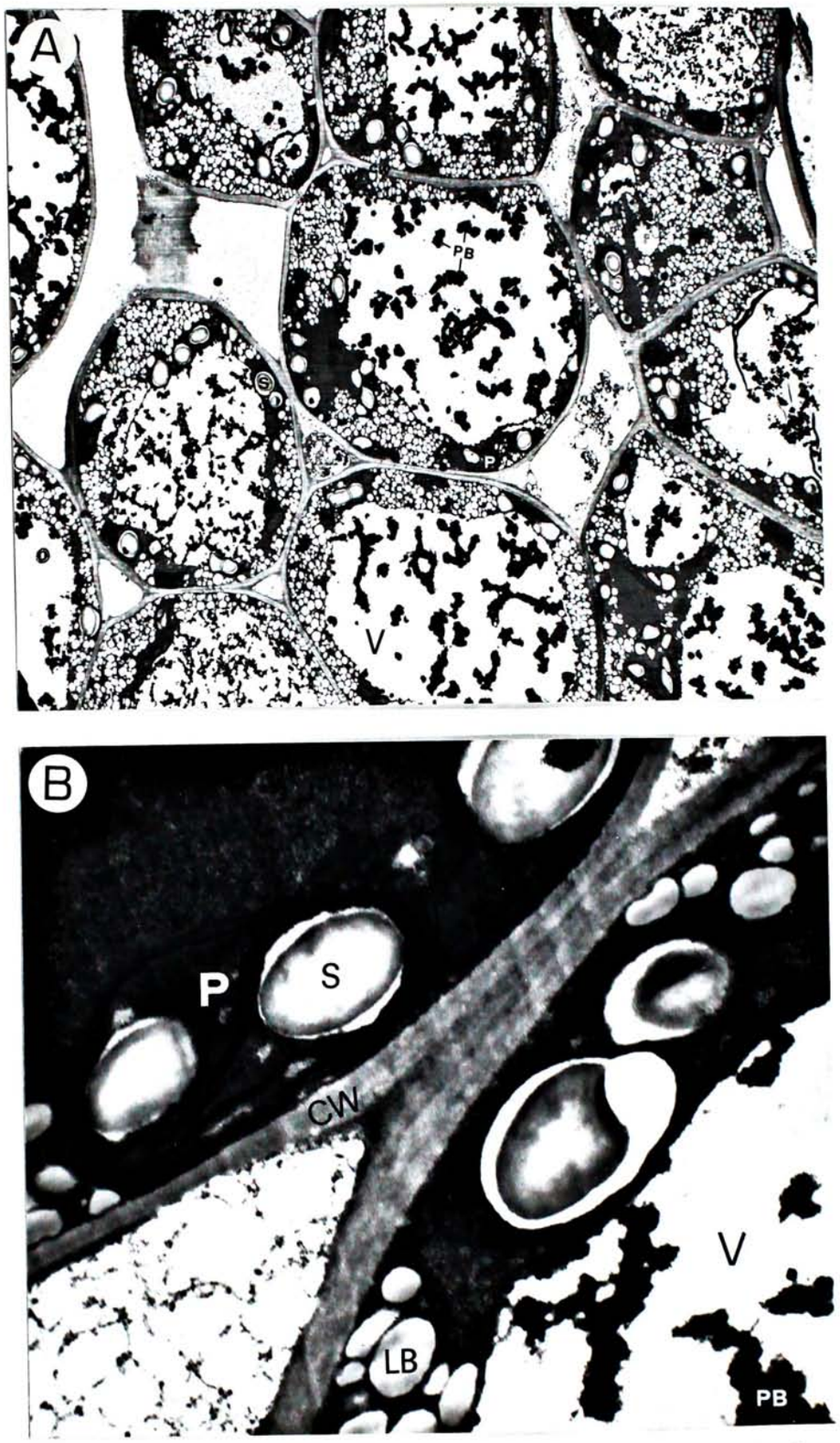
Figs. 4.53A & 4.53B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in dark and in 10^{-5}M NCS for 12 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



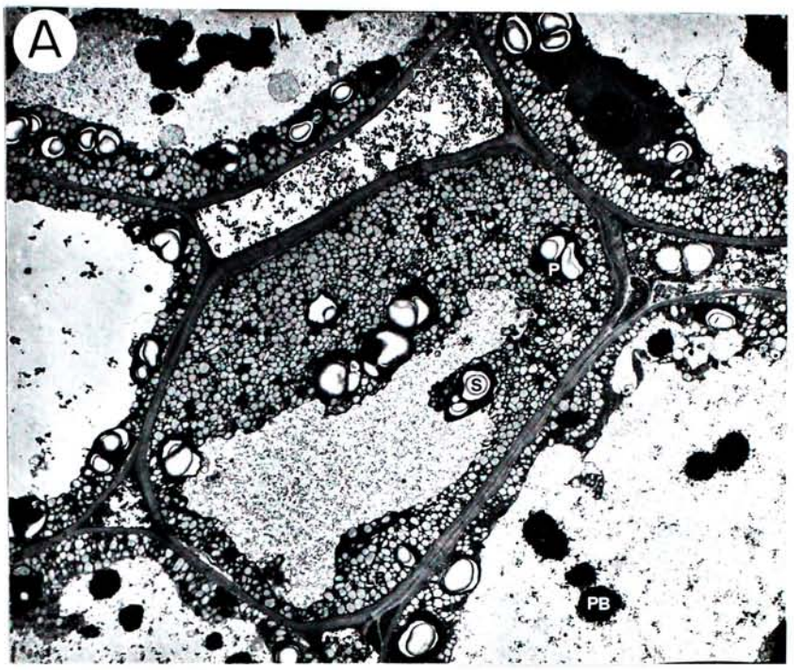
Figs. 4.54A & 4.54B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in dark and for 24 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.

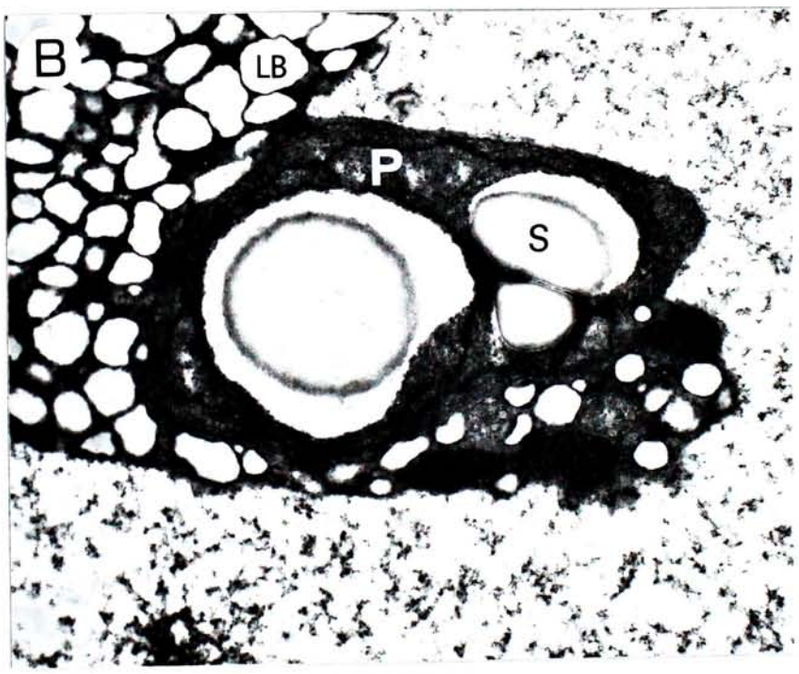


Figs. 4.55A & 4.55B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in dark and in 10^{-7}M NCS for 24 hr in light. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.



Figs. 4.56A & 4.56B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in dark and in 10^{-6}M NCS for 24 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.





Figs. 4.57A & 4.57B. Electron micrographs of excised radish cotyledonous cells incubated in water for 48 hr in dark and in 10^{-5}M NCS for 24 hr in light at 28°C. Excised radish cotyledons were obtained from radish seeds after 48 hr germination in dark at 22±2°C.
C, chloroplast; CW, cell wall; LB, lipid body; N, nucleus; P, plastid; PB, protein body; S, starch grain; V, vacuole.
A: low power, x1,800; B: high power, x18,000.

Chapter 5. Discussion

The bulb is a kind of storage organ in plants. It is composed of a stem and leaves for storage (Bryan, 1989). *Narcissus* is one of the major ornamental bulbs in temperate regions. The annual cycle of *Narcissus* is characterized by its distinct periodicity of growth and quiescence. This enables the genus in its natural habitat to compress above-ground growth within the short period between cold winter and hot, dry summers (Hanks, 1993). When slits were introduced on the *Narcissus* bulbs, slimy mucilage secreted from the cuts of the slits. It was observed if the slimy mucilages from the cuts were removed, it would accelerate the sprouting and blooming of *Narcissus*. In this study, the inhibitory substance was crystallized from the slimy mucilages and the crystal appeared yellowish (Fig. 4.7). Slimy secretions exhibited a wide range of inhibitory effects such as seed germination and seedling growth etc. (Poon, 1986). The structure of this substance was identified based on IR spectrum (Fig. 4.8), UV spectrum (Fig. 4.9), Mass spectrum (Fig. 4.10), ^{1}HNMR spectrum (Fig. 4.11) and X-ray analysis (Fig. 4.13). Its molecular weight was 307. The crystal was the same substance isolated from various species of *Narcissus* including *Narcissus tazetta* L. as reported by Piozzi et al. in 1968. This substance was first named as narciclasine by Ceriotti in 1967. It has been isolated from *Narcissus* bulb and other Amaryllidaceae plants. Narciclasine has a wide range of biological activities in medicine, such as antitumor (Fitzgerald et al., 1958) and antimitotic (Ceriotti, 1967) activities, inhibited protein synthesis (Jimenez et al., 1976), antiviral (RNA) activity (Gabrielsen et al., 1992) and antineoplastic activities (Pettit et al., 1986, 1993, 1995a,

1995b), as well as inhibitory effect on growth of wheat radicle (Ceriotti, 1967). The role of the slimy mucilage in *Narcissus* bulb might serve as protection and/or regulation to the plant itself.

In general, when a natural plant substance is observed to exert a growth-regulating activity, the first question concerned is its mode of action. Since the mode of action of most natural plant growth substances is implicated through the mediation of plant hormone, we firstly tried to determine the relationship between inhibitory effects of narciclasine and phytohormones.

In our study, it was found that NCS was a kind of very active biological substance. It had a wide range of inhibitory effects on plant growth and development (Figs. 4.14 - 4.57; Tables 4.6 -4.17). NCS strongly inhibited seed germination and seedling growth (Tables 4.6 - 4.17 and Fig. 4.14). It was interesting that 10^{-8} M NCS had no inhibitory effect on the seed germination of *Brassica parachinensis* and seemed to promote the radicle growth of *Brassica parachinensis* (Table 4.6). The biological effect of NCS on seed germination and seedling growth was very similar to that of ABA. As observed in Tables 4.9, 4.10 and Fig. 4.14, NCS exhibited a potent inhibitory effect on the germination of Chinese cabbage, rice and radish seeds. The inhibitory effect of NCS was different with various seeds. At 10^{-5}M NCS, there was approximately 98.2%, 77.8% and 73.4% inhibition on seed germination of Chinese cabbage, rice and radish, respectively. The inhibitory activity of NCS on seed germination either in Chinese cabbage or rice was a little higher than that of ABA. And the simultaneous addition of ABA can not enhance the inhibitory effect of NCS (Table 4.15).

The role of ABA in growth regulation has been studied by numerous authors (Addicott et al., 1964; Aspinall et al., 1967; Chrispeels and Varner, 1966; Milborrow, 1966,1974; Rothwell and Wain, 1954; Sivori et al., 1971). Depending on its concentration, it inhibits root elongation of *Zea mays* (Gaither et al., 1975; Pilet and Chanson, 1981), *Lens culinaris* (Gaither et al., 1975) and *Allium cepa* (De La Torre et al., 1972), but stimulates growth of excised root of *Phaseolus coccineus* (Abour-Mandour and Hartung, 1980), *Pisum sativum* (Gaither et al., 1975) and *Glycine max* (Yamaguchi and Street, 1977). In the cases of Chinese cabbage and rice, the seedling growth was also strongly inhibited by NCS or ABA (Tables 4.11, 4.12 and 4.13, 4.14). However, some differences between the effect of the two growth regulatory substances are observed. NCS action seems to be stronger than that of ABA at 10^{-5}M. In the growth of rice seedlings (Tables 4.13 and 4.14), ABA (10^{-5}M) showed more potent inhibition on the elongation of coleoptiles of rice. In contrast, NCS (10^{-5}M) had more potent inhibition on the elongation of radicles of rice. The simultaneous addition of ABA can not enhance the inhibitory effects of NCS to seedling growth, including both the radicle and the hypocotyl (Tables 4.11 and 4.12). Possibly NCS and ABA are competitive for the same site, and NCS is more effective than ABA. The seed germination and seedling growth of radish was also significantly inhibited by NCS (Fig. 4.14 and Tables 4.7, 4.8). Approximately 28.9% and 52.6% inhibition on the elongation on rice coleoptiles were observed in treatment with 10^{-6} M and 10^{-5}M NCS after incubation for 72 hr in the dark, respectively (Table 4.14). There were approximately 93.7% and 70.5% inhibition on hypocotyl elongation of Chinese cabbage and radish, treated with 10^{-6}M NCS after incubation for 72 hr or 60 hr in

the dark, respectively (Tables 4.12 and 4.8). It was clear that the elongation of hypocotyls of Chinese cabbage was more sensitive to NCS than that of radish. It appeared that this inhibitory effect of NCS was species specific. In the elongation of radicles, there were about 62.7% and 93.4% caused by NCS (10^{-6}M) after 72 hr incubation in rice and Chinese cabbage, respectively (Tables 4.11 and 4.13). The inhibition caused by 10^{-6}M NCS on the elongation of radish radicles was approximately 81.3% after 60 hr incubation (Table 4.7). It seems that the elongation of rice radicles was more sensitive to NCS than the coleoptile elongation. However, the inhibitory effect of NCS on the elongation of radicles and hypocotyls of Chinese cabbage was almost the same. This indicated that the inhibitory effect caused by NCS was both organ and species specific. The inhibitory effect of NCS on seedling growth was more remarkable than that on seed germination in Chinese cabbage and rice. There was about 26.9%, 81.2% and 70.5% inhibitions on seed germination, radicle elongation and hypocotyl elongation of radish by 10^{-6}M NCS, respectively, after 60 hr in the dark. These findings demonstrated that NCS can inhibit seed germination and seedling growth of rice, Chinese cabbage and radish. It was suggested that NCS may be a general seed germination inhibitor.

Table 4.18 showed that NCS reduced the stimulatory effect of IAA on the elongation of wheat coleoptile sections. The inhibitory effect caused by NCS lower than 10^{-5}M on the elongation of wheat coleoptile sections can be reversed by 10^{-6}M IAA. It seems to suggest that NCS is an anti-IAA substance.

As Table 4.19 presented, NCS in the concentration range strongly reduced GA activity in the barley endosperm bioassay. It has been discovered that gibberellins control the synthesis of particular enzymes. The most thoroughly investigated case is the GA-controlled synthesis of α-amylase in embryo-free seeds of barley endosperm. It was found that GA induced the increase in α-amylase activity in barley (Nicholls and Paleg, 1963) or rice (Sakoda et al., 1991). It has been indicated that the stimulation of α-amylase activity by GA is due to *de novo* synthesis of the enzyme rather than activation of a preexisting enzyme (Varner, 1964; Filner and Varner, 1967; Ho and Varner, 1974, 1978; Higgins et al., 1976). The increase in α-amylase activity is prevented by protein synthesis inhibitors and RNA synthesis inhibitors. NCS significantly inhibited the formation of α-amylase activity. It has been indicated that the antitumor activity of NCS was caused by the strong inhibitory effect on protein synthesis in eukaryotic ribosome, which was due to its interaction with the peptidyl transferase center of the large subunit of eukaryotic ribosome's (Carrasco et al., 1975, Jimenez et al., 1975). Our results suggest NCS may be a protein synthesis inhibitor.

It has been reported that cytokinins induce expansion of excised cotyledons of various species (Lethan, 1971; Narain and Laloraya, 1974). The mechanism of this response has been studied. These studies indicate that although cell division is promoted slightly, the major cause of growth in increased cell expansion results from water absorption (Gordon and Letham, 1975; Huff and Ross, 1975; Bewli and Witham, 1976; Rijven, 1976; Longo et al., 1978). From the studies of cytokinin's effect on various

cotyledons, cucumber (Harvey et al., 1974), sunflower (Servettaz et al., 1976) and watermelon (Longo et al., 1978), it appears that cytokinins accelerate the change the mode of nutrition in excised cotyledons from heterotrophic to autotrophic. In this study, NCS (10^{-6}M) significantly inhibited the stimulatory effect of BA on growth of excised radish cotyledons whether they were incubated in the light or in the dark (Tables 4.20 and 4.21). The inhibition caused by 10^{-6}M NCS on the excised radish cotyledons which were incubated in the light was about 75.6%. The inhibition caused by 10^{-6}M NCS on those excised radish cotyledons incubated in the dark was about 41.9%. It is clear that the inhibitory effect of NCS (10^{-6}M) on the expansion of excised radish cotyledons incubated in the light is stronger than that of the excised cotyledons incubated in the dark.

As shown in Table 4.22 and Table 4.23, NCS also significantly inhibited the chlorophyll or carotenoid formation of excised radish cotyledons when they were incubated in the light or in the dark for 48 hr. Carotenoid are one of the components of plastids and can be used as an index for plastid development (Longo et al., 1981). These results suggest that NCS not only inhibits the growth of excised radish cotyledons, but also repressed the chloroplast or plastid development in continuous illumination or in the dark, and the chloroplast or plastid development appeared to be more sensitivity to NCS. It seems that NCS causes growth inhibition through suppressing the action of cytokinin, but this requires further study.

The interaction between ABA and other growth regulators has been described in a great number of papers. ABA seems to an antagonist of cytokinins (Van Overbeek et al., 1968; Biddington and Thomas, 1977; Krawiarz et al., 1977) and of gibberellins (Thomas et al., 1965; Chrispeels and Varner, 1967). Treatment with cytokinins can overcome the inhibitory effect of ABA on several developmental processes like seed germination and cotyledon growth or greening (Fountain and Bewley, 1976; Krawiarz et al., 1977; Karavaiko et al., 1978). Table 4.28 and Table 4.29 showed that both BA and GA_3 stimulated while ABA inhibited the growth and greening of excised radish cotyledons in the light. The inhibition of ABA on either expanding or greening of excised radish cotyledons was partially reversed by simultaneous addition of BA or GA_3 (Tables 4.30 and 4.31). However, the inhibitory effect caused by 10^{-6}M and 10^{-5}M NCS on excised radish cotyledons expanding could hardly be reversed by BA or GA_3 (Table 4.32). ABA (10^{-6}M) did not enhance the inhibition caused by NCS on expanding or greening of excised radish cotyledons (Tables 4.32 and 4.33). The action of NCS on excised radish cotyledons expansion and greening of excised radish cotyledons is very similar to those treated with ABA. NCS was more effective than ABA in seed germination. Furthermore, the inhibition of NCS on growth or greening of excised cotyledons could hardly be reversed by BA or GA_3. On the contrary, the inhibitory effect of ABA could partially be reversed by BA or GA_3.

Cytokinins stimulate conversion of proplastids to chloroplasts in cotyledons or other tissues (Stetler and Laetsch, 1965; Harvey et al., 1974; Wozny and Szweykowska,

1975; Farineau et al., 1978). As reported by Thomas et al. in 1980, cytokinin-induced expansion of radish cotyledons did not appear to be the result of accelerated lipid breakdown or more rapid development of photosynthetic capacity. In their study, the only effect of zeatin observed was accelerated disappearance of protein bodies and appearance of the central vacuole. It is unlikely that protein degradation accounts directly for cytokinin-stimulated cell enlargement. It has been reported that the total protein content remained constant during growth of radish cotyledons (Huff and Ross, 1975; Gordon and Letham, 1975), indicating that reserve proteins are readily converted to other types of compounds (Thomas et al., 1980). From ultrastructural studies (Fig. 4.43), BA appeared to stimulate the degradation of protein bodies and lipid bodies, as well as chloroplast development of the excised radish cotyledons when they were incubated for 48 hr in the light. ABA (10^{-6}M and 10^{-5}M) (Figs. 4.44 and 4.45) inhibited the chloroplast forming and developing similar to the result obtained by Le Page-Degivry et al. in apple cotyledons (Le Page-Degivry et al., 1986). NCS significantly inhibited the degradation of protein bodies and lipid bodies, as well as chloroplast formation of the excised radish cotyledons after 48 hr in the light (Figs. 4.46-4.48). There was only a little degradation of protein bodies and lipid bodies and almost no chloroplast formation in the excised radish cotyledons treated with NCS (10^{-6}M and 10^{-5}M). The degradation of protein bodies and lipid bodies decreased with increasing NCS concentrations.

Exogenous application of cytokinin promotes expansion of cotyledons and accelerates their development from storage organs to photosynthetic organs (Longo et al.,

1979). It was found that 1 to 2 hr treatment with 10^{-4}M BA immediately after excision was as effective as a continuous exposure to the hormone for several days. And a significant response could be observed after a treatment with BA for a few minutes (Longo et al., 1978). A treatment with 10^{-5}M ABA blocked the growth and plastid development of excised watermelon cotyledons in the dark (Longo et al., 1981). Longo and his workers found that this blocking could be prevented by an initial treatment with 10^{-4}M BA for 2 hr. In this study, the effect of pretreatment with BA on growth and greening of excised radish cotyledons were also very marked. The pretreatment with 10^{-5} M BA for 4 hr was as effective as a continuous exposure to the hormone for 2 days in the expansion of excised radish cotyledons (Table 4.24). When those cotyledons incubated in BA were transferred to 10^{-6}M or 10^{-5}M NCS, the BA-promoted activity was completely blocked by both concentrations of NCS, even if the time pretreated with BA reached 4 hr.

There was inhibition on the expansion and greening of excised radish cotyledons after being pre-incubated in NCS solutions (either 10^{-6}M or 10^{-5}M) for 5 min to 4 hr, they were then transferred to water or BA (10^{-5}M) in light. Table 4.26 and Table 4.27 showed that 5 min pre-incubation in NCS solution caused inhibition in expansion as well as chl a, chl b and total chl formation in excised radish cotyledons, respectively. The longer the pretreatment times in NCS, the more inhibition on expansion and greening of excised radish cotyledons was observed. Table 4.26 showed that BA could reverse the inhibitory effect on the expansion of cotyledons caused by 10^{-6}M NCS pretreated up to 4 hr,

whereas on cotyledons pretreated with 10^{-5} M NCS, BA could only reverse the inhibitory effect caused by 10^{-5}M NCS within 1 hr.

Similar results were observed in chlorophyll accumulation (Table 4.27). Pretreatment with 10^{-5}M NCS caused greater inhibition on the chlorophyll accumulation than 10^{-6}M NCS. The inhibition on chlorophyll accumulation caused by pretreatment with 10^{-6}M NCS from 5 min to 30 min could be reversed by subsequent transfer to BA solution for 48 hr in light. The inhibition caused by 10^{-6}M NCS pretreated cotyledons from 1 hr to 4 hr could partially be reversed by subsequent transfer to BA solution for 48 hr in light. However, the incubation in BA for 48 hr in the light could only reverse the inhibition of a 5 min pretreated with 10^{-5}M NCS.

As shown in Fig. 4.19 and Fig. 4.20, the experiments involving two-stage incubations of excised radish cotyledons in water, incubation in water for 12 hr or 24 hr followed by being transferred to NCS solutions, showed that the NCS-induced (10^{-5}M and 10^{-6}M) inhibitory effects on growth of excised cotyledons could significantly be reduced by preincubation in water for 12 hr and 24 hr in the light, especially in 24 hr preincubation in water. Due to the preincubation in water for 24 hr, the inhibition caused by 10^{-6}M or 10^{-5}M NCS on growth of excised radish cotyledons was decreased from 80.2% to 5.9% or from 92.1% to 19.2% after incubation in 10^{-6}M or 10^{-5}M NCS for 24 hr in light, respectively. In the chlorophyll formation, only the preincubation in water for 24 hr could reduce the inhibition of NCS both 10^{-6}M and 10^{-5}M after they were transferred

to NCS solutions for 12 hr. It is interesting that the chlorophyll contents of excised radish cotyledons decreased in prolonging incubation in NCS solutions for 24 hr. These experiments thus suggest that the inhibitory effects of NCS differed according to the physiological state of the material; when NCS was applied to a newly excised cotyledon, NCS caused severe inhibition on growth and greening of excised cotyledons, but when NCS was applied to the excised cotyledons which were incubated in water for 24 hr, it no longer strongly prevents growth and causes only partial inhibition of chlorophyll synthesis. It is possible that NCS exhibits more inhibition to plastid differentiation and consequently chlorophyll production than that of the breakdown of storage materials. In addition, it was very interesting that the chlorophyll accumulation of those cotyledons transferred to two kinds of NCS concentrations after growing in water for 24 hr increased and then decreased. These results suggest that NCS probably not only inhibited chlorophyll production of excised radish cotyledons growing in light, but also induced the degradation of formed chlorophyll.

From electron microscopic studies, only marked degradation of protein bodies was observed in the intracellular space of excised radish cotyledon incubated in water for 12 hr in light (Fig. 4.30). At 24 hr (Fig. 4.33), a significant number of chloroplasts appeared, however no typical granum structure was established. NCS (10^{-7}M) strongly inhibited the chloroplast formation and development of excised cotyledons during incubation for 48 hr in light (Figs. 4.31, 4.34, 4.37 and 4.40). At 24 hr, some chloroplasts appeared and also no granum structure was established (Fig. 4.34). NCS (10^{-6}M) totally

blocked the chloroplast formation of excised radish cotyledons during incubation for 48 hr in light (Fig. 4.32, 4.35, 4.38 and 4.41). When 10^{-6}M NCS was applied after incubation in water for 24 hr in light, the plastid differentiation appeared. It could only partially inhibit the expansion and chlorophyll synthesis in excised cotyledons. Conversely, if it was applied from the initial of excision of radish cotyledons after germinating for 48 hr in the dark, 10^{-6}M NCS could significantly inhibit the growth, plastid differentiation and, consequently, chlorophyll synthesis of excised cotyledons.

If the excised cotyledons were preincubated with water in the dark for different times, the inhibitory effect of NCS on the growth of those cotyledons was also markedly reduced (Fig.4.21). The inhibition by NCS (10^{-7}M, 10^{-6}M and 10^{-5}M) was approximately 11%, 20% and 35%, respectively, when the cotyledons were incubated in the light for 12hr after preincubating in water for 48hr in the dark. Conversely, there was about 39%, 53% and 57% inhibition of NCS (10^{-7}M, 10^{-6}M and 10^{-5}M) on the excised cotyledons without preincubating in the dark. But the greening of them was still strongly inhibited by 10^{-6}M and 10^{-5}M NCS as in those cotyledons without preincubating with water in the dark (Fig. 4.22).

The ultrastructural observation indicated the cotyledons preincubated with water in dark for 48 hr showed obvious degradation of protein and lipid bodies, and some etioplasts with large starch grains (Fig. 4.49). When they were exposed to light for 12 hr, grana were formed in plastids of control cotyledons (Fig. 4.50). When they were

transferred to different concentrations of NCS (10^{-6} and 10^{-5}M) and also exposed to light for 12 hr, there were almost no plastid development or grana forming in the excised cotyledons at higher concentrations of NCS (10^{-6} and 10^{-5}M) (Figs. 4.52 and 4.53), even though they were exposed to light up to 24 hr (Figs. 4.56 and 4.57). These results suggest that either reserves breakdown or the development of photosynthetic organs are significantly inhibited by NCS. It seems that NCS is more effective inhibiting on the chloroplast differentiation and followed chlorophyll synthesis than the breakdown of reserve materials.

Tetrapyrrole compounds are essential substances of life. They include hemes, which serve as prosthetic groups of respiratory enzymes, and chlorophylls, which are the major photosynthetic light-harvesting pigments (Senge, 1993). δ-Aminolevulinic acid (ALA) is the precursor of tetrepyrrols in plants and animals (Beale, 1978, Richards 1993). The formation of ALA provides the basic control point in the multistep pathway of tetrapyrrole biosynthesis, especially in the case of chlorophyll during dark/light transitions (Beale and Weinstein, 1990). It has been indicated that chlorophyll synthesis involves multienzymes and multisteps (Granick, 1967). Exogenous addition of ALA to dark-grown seedlings leads to the accumulation of protochlorophyllide (Sisler and Klein, 1963; Nadler and Granick, 1970; Castelfranco et al., 1974), suggesting that all of the enzymes required for the synthesis of protochlorophyllide from ALA are present in significant quantities in etiolated tissue and that ALA formation is the rate-limiting step in chlorophyll biosynthesis in dark-grown plants. When etiolated seedlings are first

illuminated, the initial photoreduction of protochlorophyllide to chlorophyllide is not immediately followed by maximal chlorophyll synthesis. Instead, there is a lag phase during which the enzyme system responsible for ALA synthesis appears to be formed de novo. Indeed, after the lag phase, the rate of chlorophyll synthesis is enhanced and accompanied by a commensurate increase in level of ALA-forming activity (Castelfranco et al., 1974). Treatment of etiolated leaves with ALA increases the concentration of protochlorophyll (Granick, 1959, Sumdqvist , 1969, Egneus and Sundqvist, 1970, Sundqvist and Klockare, 1975).

Levulinic acid (LA), a competitive inhibitor of ALA dehydratase (Beale and Castelfranco, 1974; Klein et al., 1975), inhibits the conversion of ALA to chlorophyll, causing the accumulation of ALA. Application of LA to a developed green plant will not have much impact as its photosynthetic apparatus is already well developed. The rate of chlorophyll synthesis in a mature plant is also significantly lower than that of a developing and greening seedling. Therefore, the studies chlorophyll synthesis or chloroplast development by exposing etiolated plant materials to light in the presence of the LA are needed (Jilani et al., 1996). In the presence of LA, the measurement of ALA accumulation can serve as a useful tool for studying ALA synthesis (Klein et al., 1975). It has been suggested that the formation of ALA plays a central role in the metabolic control of chlorophyll biosynthesis (Bogorad, 1966). The demonstration of the formation of ALA requires light in higher plants thus makes it possible to study more directly problems concerning the control mechanisms of chlorophyll synthesis.

The presence of LA at various concentrations exhibited the accumulation of ALA in etiolated wheat leaves (Fig. 4.24). LA at concentrations higher than 25mM reduced ALA accumulation. This result is similar to that in barley leaves (Beale and Castelfranco, 1974) and bean leaves (Klein et al., 1975). The reason for this decrease is not clear. It might be due to the feed-back inhibition of ALA, as reported for tissue cultures of tobacco (Schneider, 1973), the toxic effects of the high concentrations of ALA , or the damage caused by higher concentrations of LA. In the presence of 25mM LA, NCS inhibited the accumulations of ALA and chlorophyll, especially at 10^{-6}M NCS (Fig. 4.25). Nadler and Granick (1970) indicated that in 7- to 10-day-old leaves of etiolated barley, all of the enzymes that convert δ-aminolevulinic acid to chlorophyll were nonlimiting during the first 6 to 12 hr of illumination, even in the presence of inhibitors of protein synthesis. The limiting activity for chlorophyll synthesis appeared to be a protein (or proteins) related to the synthesis of δ-aminolevulinic acid, presumably δ-aminolevulinic acid synthase (Nadler and Granick 1970). Acceleration of chlorophyll formation by light was not blocked by inhibitors of nucleic acid synthesis, but by inhibitors of protein synthesis. As a result, they proposed a model for control of chlorophyll synthesis based on a light-induced activation at the translational level of the synthesis of proteins forming δ-aminolevulinic acid.

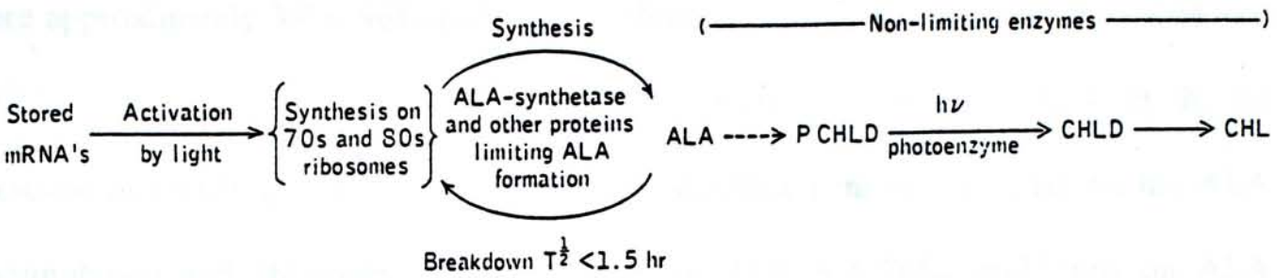


Fig. 5.1 A tentative working model for the control of chlorophyll biosynthesis in barley (Nadler and Granick, 1970)

They proposed that illumination could activate stored mRNA to make the required proteins for ALA synthesis. Synthesis of the proteins requires the participation of 80S ribosomes of the cytoplasm and 70S ribosomes of the plastids. Once ALA is made, it is converted to chlorophyll by other enzymes of the biosynthetic chain; these enzymes are nonlimiting. In this model, chlorophyll synthesis is controlled by the activity of ALA synthetase. In this investigation, NCS significantly blocked the chlorophyll synthesis of etiolated wheat leaves exposed to light (Fig. 4.23). The inhibition caused by NCS on chlorophyll synthesis increased with increasing concentrations. The total chlorophyll content in the control increased 28.8 times and 108.6 times after exposing to light for 12

hr and 48 hr, respectively. At the presence of NCS (10^{-7}M, 10^{-6}M and 10^{-5}M), the total chlorophyll content increased only 21.9, 2.6 and 0.9 times, respectively, after 12 hr in the light. About 71.5, 4.6 and 1.6 times of chlorophyll contents at presence of 10^{-7}M, 10^{-6}M and 10^{-5}M NCS, respectively, were observed after incubation for 48 hr in light . There were approximately 34%, 96% and 99% inhibitions caused by NCS (10^{-7}M, 10^{-6}M and 10^{-5}M, respectively) on the total chlorophyll contents after 48 hr in the light. In the presence of 25mM LA, 10^{-7}M NCS exhibited significant inhibitory effect on the ALA accumulation and chlorophyll. There was about 23% and 76% inhibitions on ALA accumulation by NCS at 10^{-7}M and 10^{-6}M, respectively, after 16 hr in the light. NCS (10^{-6} M) completely blocked ALA accumulation after 24 hr in the light. This suggests that NCS might be a protein synthesis inhibitor in chlorophyll synthesis in light as in the inhibition on GA-induced α-amylase production in barley embryo-free seeds. The inhibitory effect of NCS on chlorophyll synthesis of etiolated wheat leaves exposed to light was possibly caused by inhibiting ALA formation. It has been indicated that the antitumor activity of NCS was caused by the strong inhibitory effect on protein synthesis in ribosome, which is due to its interaction with the peptidyl transferase center of the large subunit of ribosomes (Carrasco et al., 1975; Jimenez et al., 1975).

Glyoxysomes play a very important role in the postgerminative growth of seeds containing high content of oil. Glyoxysomes and peroxisomes are two important functional cell organelles in oilseed germination. These organelles contain all the enzymes for the glyoxylate-cycle and the β-oxidation (Breidenbach et al., 1967; Cooper

and Beevers, 1969; Hutton and Stumpt, 1969). These enzymes catalyze the net conversion of fatty acids to succinate and are mainly responsible for the suitability of plants to utilize fatty acids as a carbon source (Beevers, 1980; Trelease, 1984). These two classes of cell organelles have been the subject of many reviews (Tolber, 1971, 1981; Beevers, 1979; Kindl and Lazarow, 1982; Huang et al., 1983; Lord and Roberts, 1983; Trelease, 1984; Lazarow and Fujiki, 1985; Borst, 1989; Olsen and Harada, 1991, 1995; Kindl, 1992; Van den Bosch, 1992; Subranmani, 1993).

Isocitrate lyase and malate synthase are the key enzymes of the glyoxylate pathway (Carpenter and Beevers, 1959; Yamamoto and Beevers, 1960; Presley and Fowden, 1965; Mori and Nakamura, 1989). Hydroxypyruvate reductase and glycolate oxidase are the key enzymes of the peroxisomes (McGregor 1969; Tolbert et al., 1970; Kagawa et al., 1973; Kagawa and Beevers, 1975; Becker et al., 1978). Studies with a variety of species have established a characteristic developmental pattern for the key enzymes of the glyoxylate cycle during the early postgerminative growth of light-grown seedlings containing high fatty acids contents. Little or no isocitrate lyase or malate synthase is detectable in dry seeds (Marcus and Velasco, 1960), but both enzymes increase markedly in activity shortly after germination, reaching a peak within a few days, and then decline rapidly as lipid reserves are depleted (Carpenter and Beevers, 1959; Ching, 1970; Firenzuoli et al., 1968; Karow and Mohr, 1967; Lado et al., 1968; Lee et al., 1964; Longo and Longo, 1970; Marcus and Velasco, 1960; McGregor and Beevers, 1969; Trelease et al., 1970a, b). The postgerminative increase in activity of isocitrate

lyase or malate synthetase is known to result from *de novo* enzyme synthesis both in endosperms (Lado et al., 1968) and in cotyledons (Gientka and Cherry, 1968; Hock and Beevers, 1966; Longo, 1968). The cell organelles of some high oil-content cotyledons are interesting to study, since the cotyledons grown in light expand and differentiate into photosynthetic organs after depletion of lipid stores. In such cotyledons, the activities of glyoxysomal enzymes decrease while enzyme activities of leaf peroxisomes increase correspondingly (Gruber et al., 1970; Schnarrenberger et a., 1971; Gerhardt, 1973; Kagawa et al, 1973; Drumm and Schopfer, 1974; Kagawa and Beevers, 1975).

As shown in Fig. 4.26, NCS inhibited isocitrate lyase activity. In the control treatment, the activity of isocitrate lyase reached a maximum at 24 hr, and then decreased. This pattern was consistent with that occurring in cotyledons of radish (Thomas et al., 1980) and other species (Servettaz et al., 1976; Theimer et al., 1976; Longo et al., 1978). In NCS-treated (10^{-7}M) cotyledons, this enzyme activity also reached a maximum at 24 hr. However, the activity of isocitrate lyase was only about 79% of the control. The peak of isocitrate lyase activity was observed in excised radish cotyledons treated with NCS (10^{-6}M) at 36 hr and not at 24 hr. It was about 56% of the maximum activity of the control, while 10^{-5}M NCS completely inhibited the production of isocitrate lyase activity. It is obvious that NCS significantly inhibited the isocitrate lyase activity during the period of excised cotyledons grown in light for 48 hr. This might be either due to direct inhibition of the activity of the enzyme and/or the synthesis of this enzyme.

The effect of different concentrations of NCS on the activity of hydroxypyruvate reductase of excised radish cotyledons was very similar to that of their growth (Fig. 4.28). NCS (10^{-5}M) totally inhibited the hydroxypyruvate reductase activity of excised radish cotyledons. The inhibition caused by 10^{-7}M and 10^{-6}M NCS on hydroxypyruvate reductase activity was approximately 4.9% and 77%, respectively, after incubation for 48 hr in the light (Figs. 4.27 and 4.28). The inhibitory effect of NCS on growth of excised cotyledons incubated in light was more effective than that of excised cotyledons incubated in dark. It is possible that the more effective inhibition of NCS on growth of excised cotyledons incubated in light was mainly due to the blocking of chlorophyll formation and hydroxypyruvate reductase activity.

Chapter 6. Conclusions

Previous studies have claimed the presence of bioactive substances in bulbs of Amaryllidaceae plants. In this study, the inhibitory substance was crystallized from the slimy mucilages and the crystal appears yellowish (Fig. 4.7). The structure of this substance was identified based on IR. spectrum, UV spectrum, Mass spectrum, ^{1}HNMR spectrum and X-ray analysis. Its molecular weight was 307. This crystal-narciclasine as named by Ceriotti (1967)-turn out to be the same substance isolated from various species of *Narcissus* including *Narcissus tazetta* L. as reported by Piozzi et al. in 1968.

NCS had a wide range of inhibitory effects on plant growth and development. It strongly inhibited seed germination and the growth of seedlings. The biological effect of NCS on seed germination and the growth of seedlings was very similar to those of ABA. NCS exhibited a potent inhibitory effect on the germination of Chinese cabbage, rice and radish seeds. The inhibitory effects of NCS differed with regard to the type of seeds. The inhibitory activity of NCS to seed germination either in Chinese cabbage or rice was slightly higher than that of ABA. The simultaneous addition of ABA can not enhance the inhibitory effect of NCS. In the case of Chinese cabbage and rice, the seedling growth was also strongly inhibited by NCS. NCS action seemed to be stronger than that of ABA at 10^{-5} M. In the growing of rice seedlings, ABA (10^{-5}M) showed more inhibition on the elongation of coleoptile of rice as compare with NCS. In contrast, NCS (10^{-5}M) had more potent inhibition on the elongation of radicle of rice. The simultaneous addition of ABA

and NCS did not enhance the inhibitory effect of NCS on seedling growth, either radicle or hypocotyl. It might possibly be that NCS and ABA compete for the same site, and NCS is more effective than ABA. In radish, the seed germination and seedling growth were also significantly inhibited by NCS. It appeared that this inhibitory effect of NCS was species specific. It seemed that the elongation of rice radicle was more sensitive to NCS than the coleoptile elongation. However, the inhibitory effect of NCS on the elongation of radicle and hypocotyl of Chinese cabbage was almost the same. These results indicated that the inhibitory effect caused by NCS was both organ and species specific. The inhibitory effect of NCS on seedlings growth was more remarkable than that on seed germination in Chinese cabbage and rice. These findings demonstrated that narciclasine can inhibit seed germination and seedling growth of rice, Chinese cabbage and radish. It was thus suggested that narciclasine may be a general seed germination inhibitor.

In the study of the interaction of NCS with phytohormones, NCS reduced the stimulatory effect of IAA on the elongation of wheat coleoptile sections. The inhibitory effect on the elongation of wheat coleoptile sections caused by lower than 10^{-5}M NCS can be reversed by 10^{-6}M IAA. From these data, NCS could be considered as an anti-IAA substance. GA-induced α-amylase activity in the barley endosperm bioassay and BA-promoted activity of the expansion and greening of excised radish cotyledons were also strongly blocked by NCS. It has been discovered that gibberellins control the synthesis of particular enzymes, because the increase in α-amylase activity is prevented by protein

synthesis inhibitors and RNA synthesis inhibitors. NCS significantly inhibited the formation of α-amylase activity. This result suggested that NCS might be a protein synthesis inhibitor. On the other hand, NCS also significantly inhibited the chlorophyll or carotenoid formation of excised radish cotyledons when they were incubated in the light or in the dark, which showed that NCS not only inhibited the growth of excised radish cotyledons, but also repressed the chloroplast or plastid development in continuous illumination or in the dark. In addition, the chloroplast or plastid development appeared to have more sensitivity to NCS. Consequently, NCS might cause growth inhibition through suppressing the action of cytokinin. The action of NCS in excised radish cotyledons expansion and greening of excised radish cotyledons was very similar to those treated with ABA. Furthermore, the inhibition of NCS on growth or greening of excised cotyledons was hardly reversed by BA or GA_3. On the contrary, the inhibitory effect of ABA could be partially reversed by BA or GA_3.

The effects of pretreatment with BA on growth and greening of excised radish cotyledons were very strong. The pretreatment with 10^{-5}M BA for 4 hr was as active as a continuous exposure to the hormone for 2 days in the expansion of excised radish cotyledons. The BA-promoted activity was completely blocked by both concentrations of NCS (10^{-6} and 10^{-5} M), even if the pretreatment time with BA reach 4 hr. Inhibition on the expansion and greening of excised radish cotyledons occurred after they were preincubated in NCS solutions (either 10^{-6}M or 10^{-5}M) for 5 min to 4 hr, and then transferred to water or BA (10^{-5}M) in light. The longer the pretreatment times in NCS, the

more inhibition on expansion and greening of excised radish cotyledons was observed. BA could reverse the inhibitory effect caused by 10^{-6}M NCS on the expansion of excised radish cotyledons that were pretreated up to 4 hr, whereas cotyledons pretreated with 10^{-5} M NCS, BA could only reverse the inhibitory effect caused by 10^{-5}M NCS within 1 hr. The inhibition on chlorophyll accumulation caused by pretreatment with 10^{-6}M NCS from 5 min to 30 min could be reversed by being subsequently transferred to BA solution for 48 hr in light. The inhibition caused by 10^{-6}M NCS pretreated from 1 hr to 4 hr could partially be reversed by being subsequently transferred to BA solution in light. However, the incubation in BA for 48 hr in the light could only reverse the inhibition of a 5 min pretreatment with 10^{-5}M NCS on chlorophyll formation.

The following experiments on the two-stage incubations of excised radish cotyledons in water, incubation in water for 12 hr or 24 hr and then in NCS solutions, showed that the NCS-induced (10^{-5}M and 10^{-6}M) inhibitory effects on growth of excised cotyledons could significantly be reduced by preincubation in water for 12 hr and 24 hr in the light, especially in 24 hr preincubation in water. In the chlorophyll formation, only the preincubation in water for 24 hr could reduce the inhibition of NCS both 10^{-6}M and 10^{-5}M after they were transferred to NCS solutions for 12 hr. It is interesting to note that the chlorophyll contents of excised radish cotyledons decreased in prolonging incubation in NCS solutions for 24 hr. The results suggested that the inhibitory effects of NCS differed according to the physiological state of the material; when NCS was applied to a newly excised cotyledon, NCS caused severe inhibition of growth and greening of

excised cotyledons, but when NCS was applied to the excised cotyledons which were incubated in water for 24 hr, it no longer strongly prevents growth and caused only partial inhibition of chlorophyll synthesis. It is possible that NCS exhibited more inhibition on plastid differentiation and accordingly on chlorophyll production than that of the breakdown of storage materials. In addition, the chlorophyll accumulation of those cotyledons transferred to two kinds of NCS concentrations after growing in water for 24 hr increased first and then decreased. These results suggested that NCS probably not only inhibited chlorophyll production of excised radish cotyledons growing in light, but also induced the degradation of formed chlorophyll. If the excised cotyledons were preincubated with water in the dark for different time intervals, the inhibitory effect of NCS on the growth of those cotyledons was markedly reduced. But the greening of them was still strongly inhibited by 10^{-6}M and 10^{-5}M NCS as in those cotyledons without preincubating in water under the dark.

It has been suggested that the formation of ALA plays a central role in the metabolic control of chlorophyll biosynthesis. The demonstration of the formation of ALA depending light in higher plants makes it possible to study more directly problems concerning the control mechanisms of chlorophyll synthesis. Levulinic acid (LA) is a competitive inhibitor of ALA dehydratase. It inhibits the conversion of ALA to chlorophyll, resulting in the accumulation of ALA. In the presence of 25mM LA, NCS inhibited the accumulations of ALA and chlorophyll, especially at 10^{-6}M NCS. The limiting activity for chlorophyll synthesis appeared to be a protein (or proteins) related to

the synthesis of δ-aminolevulinic acid, presumably δ-aminolevulinic acid synthetase. And the acceleration of chlorophyll formation by light is not blocked by inhibitors of nucleic acid synthesis, but by inhibitors of protein synthesis. NCS significantly inhibited the chlorophyll synthesis of etiolated wheat leaves exposed to light. The inhibition caused by NCS on chlorophyll synthesis increased with the increase of concentrations. It suggested that NCS might be a protein synthesis inhibitor in chlorophyll synthesis in light as in the inhibition on GA-induced α-amylase production in barley half-seed. The inhibitory effect of NCS on chlorophyll synthesis of etiolated wheat leaves exposed to light was possibly caused by inhibition of ALA synthase.

Plants which store lipid in the endosperm or cotyledons of the seed convert this lipid into carbohydrate via the glyoxylate cycle during early stage of germination. When the fatty cotyledons grow in the light, the specific activities of glyoxysomal enzymes decrease while enzyme activities of leaf peroxisomes increase correspondingly. Isocitrate lyase is a key enzyme of the glyoxylate pathway and hydroxypyruvate reductase is an enzyme characteristic of leaf peroxisomes. NCS significantly inhibited the isocitrate lyase activity during the period of excised cotyledons growth in light for 48 hr. These inhibitions were either on the activity of the enzyme directly and/or the synthesis of this enzyme. The effects of different concentrations of NCS on activity of hydroxypyruvate reductase of excised radish cotyledons was very similar to that of their growth. NCS (10^{-5} M) completely inhibited the hydroxypyruvate reductase activity of excised radish cotyledons. The inhibitory effect of NCS on growth of excised cotyledons incubated in

light was more effective than that of excised cotyledons incubated in dark. It was possible that the more effective inhibition of NCS on growth of excised cotyledons incubated in light was mainly due to the blocking of chlorophyll formation and hydroxypyruvate reductase activity.

The electron microscopic studies indicated that the cotyledons preincubated with water in the dark for 48hr exhibited obvious degradation of protein and lipid bodies, and some etioplasts with large starch granules. When they were exposed to light for 12 hr, grana had formed in the plastids of control cotyledons. If they were transferred to different concentrations of NCS and exposed to light for 12 hr as well, there were almost no progress on plastid development and grana forming in the excised cotyledons. It followed that either reserves breakdown or the development of photosynthetic organ was significantly inhibited by NCS, whereas NCS applied more efficient inhibition to chloroplast differentiation and the following chlorophyll synthesis. From ultrastructural studies, BA appeared to stimulate the degradation of protein bodies and lipid bodies, as well as chloroplast development of the excised radish cotyledons when they were incubated 48 hr in the light. ABA (10^{-6}M and 10^{-5}M) inhibited the chloroplast forming and developing. NCS significantly inhibited the degradation of protein bodies and lipid bodies, as well as chloroplast formation of the excised radish cotyledons after 48 hr in the light. There were only a little degradation of protein bodies and lipid bodies and almost no chloroplast formation in the excised radish cotyledons treated with NCS. The degradation of protein bodies and lipid bodies decreased with increasing in NCS

concentrations. Only marked degradation of protein bodies was observed in the intracellular space of excised radish cotyledon incubated in water for 12 hr in light. At 24 hr, significant number of the chloroplasts appeared, however no typical granum structure was established. When 10^{-6}M NCS was applied after incubation in water for 24 hr in light, the plastid differentiation had appeared, it could only partially inhibit the expansion and the chlorophyll synthesis of excised cotyledons. Conversely, if it was applied from the initial stage of excision of radish cotyledons after germinating for 48 hr in the dark, 10^{-6}M NCS could significantly inhibit the growth as well as plastid differentiation and, subsequently, chlorophyll synthesis of excised cotyledons.

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